MAR 23 1922

VOLUME VII NUMBER 2

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARCH, 1922

EDITOR-IN-CHIEF
C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

PUBLISHED BIMONTHLY
WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

Entered as second-class matter April 21, 1916, at the postoffice at Baltimore, Maryland, under the Act of August 24, 1912. Acceptance for mailing at special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized on July 13, 1918

Copyright 1922, Williams and Wilkins Company

Price, net { $5.00 per volume, United States, Mexico, Cuba
$5.25 per volume, Canada
$5.50 per Volume, other countries

Made in United States of America

The actual direct benefits from Gastron are a known quantity,

Unmistakably experienced by the patient, observed by the physician—in disorders of gastric function.

Inevitably the better digestion promotes better nutrition, strengthens resistance, encourages and heartens the patient, thus promotes a condition of body and a state of mind conducive to restoration.

In these circumstances there is an appeal for a wide application of Gastron; far-reaching indeed be its ultimate good effects.

Fairchild Bros. & Foster
NEW YORK

PFANSTIEHL CHEMICALS

THE STANDARD FOR RESEARCH

WE now offer 600 special products including *rare sugars, amino acids, rare earths, blood-test solutions,* and a growing list of *laboratory reagents* under analyzed labels.

The Standard of Pfanstiehl conforms to the highest published at home or abroad.

Write for list of Pfanstiehl Chemicals

SPECIAL CHEMICALS COMPANY
MAKERS OF FINE CHEMICAL INTERMEDIATES OF ANY KIND
HIGHLAND PARK, ILL.

OUR SOCIETY[1]

F. C. HARRISON

Principal, Macdonald College, P. Q., Canada

INTRODUCTION

The constitution of our Society states that the object of the Society shall be the promotion of the science of bacteriology, the bringing together of American bacteriologists, the demonstration and discussion of bacteriological methods, and the consideration of subjects of common interest: Thus, in accord with other scientific societies, its ultimate object is to make life a better thing than it is, to help in the service of man, and to attempt in some small measure to attain what Aristotle in Book III of *The Republic* has expressed that:

> Our youth will dwell in a land of health, amid fair sights and sounds and receive the good in everything; and beauty, the effluence of fair works, shall flow into the eye and ear like a health giving breeze from a pure region and insensibly draw the soul from earliest years into likeness and sympathy with the beauty of reason.

To accomplish these benefits, our work lies ready to our hands, but our strength may be the greater and our faith the firmer if we spare a moment from present toils to look back upon the achievements of the past, to gather strength and encouragement before confronting the future.

The Society was founded in 1900, under the presidency of the late Prof. W. T. Sedgwick, a great teacher, an inspiring investigator and a kindly spirit that radiated good will and courteous consideration to others. In the two decades just past the Society has increased in stature and in wisdom and in favor with fellow workers.

[1] Address of the President at the Twenty-third Annual Meeting of the Society of American Bacteriologists.

The membership has grown from fifty in 1900 to over one thousand in 1921. The Society has tasted the joy that springs from labor, and perhaps its greatest achievement has been the establishment of the JOURNAL and ABSTRACTS which are to our workers ports and happy havens indeed. Further achievements of happy memory and daily use to the teacher and investigator are the Society's card, the standard methods of many routine proceedings; some order out of the chaos of bacteriological nomenclature; and the commencement of work on standardizing materials and methods.

The recital of the past is an augury for the future. Can we, at this time, suggest a policy for our Society? Are we able to plan a coöperation of efforts which will interest all members, for each to give something of himself for the good of the Society, and for the benefit of human life and effort?

"The keen spirit seizes the prompt occasion—makes the thought start into instant action, and at once plans and performs, resolves and executes."

May I, therefore, take this opportunity of placing before you a few thoughts regarding the future.

I. STEADY CAMPAIGN FOR MEMBERSHIP, AND THE ESTABLISHMENT WHERE POSSIBLE OF LOCAL BRANCHES

Thanks to the activity of Dr. Ayers and his committee, there has been this year a large increase in membership. But we must have more, if the plans outlined for a larger journal are to be carried out; a large membership is fundamental and when obtained many other things will follow in consequence.

We have started a number of local branches. No scheme offers better prospects of success for keeping the interest in the Society between annual meetings. Every endeavour should be made to increase these local organizations for, besides interest, they afford opportunity for social meetings, arouse a feeling of professional solidarity and permit of more frequent contributions to our science. Further, by enlarging the field from which members are drawn, the danger of narrow specialization is avoided, and members of the local branches will be given a broader outlook.

II. IMPROVEMENT OF THE JOURNAL AND ABSTRACTS

When so much has been done in establishing these two important journals and bringing them to their present state of excellence, any drastic criticism would be a task of supererogation. I mention this matter, however, because our Secretary, in his circular letter dated October 25, 1921, stated that the Editor must have more space in order that papers offered for publication may appear more promptly. In order to make the JOURNAL OF BACTERIOLOGY a monthly periodical, there must be an increase of membership to 1500, or else other means must be adopted, and several alternate suggestions are set forth.

All of us would like to see the size of the JOURNAL increased, and regular publication guaranteed; these benefits can best be obtained only by an increase of membership. If our publications maintain a high standard of excellence, their worth will ensure more subscriptions from foreign countries.

Each member should be personally interested in the success of these journals, and should see that they attain a wider field of usefulness. Judicious and frequent suggestions to libraries that do not take them, bringing them to the notice of medical men and others interested in cognate subjects would help to advertise and undoubtedly secure subscribers.

III. PROVISION FOR CRITIQUES AND RÉSUMÉS

ABSTRACTS at present is filled with references and short résumés of work done by bacteriologists all over the world.

I suggest that it would be of considerable interest to arrange for critiques or comprehensive résumés on many subjects, to be written by students of particular groups. For example: a comprehensive summary of the literature on the decomposition of cellulose; botulism; the carrier of infection, etc. Such reviews would be of great value to students, save much time in hunting up references, and bring the subject up to date. If written in a judicial spirit, the writer would be able to size up the situation, and give an appreciation of the subject as a whole.

If possible, such contributors should be paid.

IV. CARD INDEX COMPILED FROM ABSTRACTS

The Library of Congress prepares and issues a card index of books and periodicals. The United States Office of Experiment Stations issues a card index of all its publications and that of the experiment stations. Would it be possible, and would it fill a need, if the editors of ABSTRACTS prepared a card index of the papers abstracted? The basis of subscription would have to be the individual card. Laboratories might arrange to subscribe according to the titles and sub-titles of the table of contents of ABSTRACTS.

General bacteriology should interest all. Agricultural colleges would naturally desire the card index for dairy, soil, and plant bacteriology. Public health laboratories should be interested in water, sewage and food bacteriology and health board laboratory methods and so on.

Prompt service as regular as the issue of ABSTRACTS would be possible.

How many would be willing to subscribe for such a service?

V. COÖPERATION BETWEEN OUR SOCIETY AND THE AMERICAN PUBLIC HEALTH ASSOCIATION

In some regards our Society and the laboratory section of the American Public Health Association cover similar subjects. Both organizations have attempted to standardise technique and methods, both have published so-called standard methods. Members of our Society have been prominent in the American Public Health Association and vice versa. I suggest that some form of coöperation be instituted which would prevent any useless duplication of work. The two societies should work together, for in unity there is strength, and projects to be initiated by each might well be considered jointly, not necessarily by the whole society in convention, but at any rate by responsible committees of each.

VI. A COMPREHENSIVE STUDY OF METHODS AND MATERIALS

Valuable work has been done by committees of the Society who have given their labor and time ungrudgingly in order to work out methods or examine materials. I mention this here because I believe that a more comprehensive survey of methods and material would be of great value. In biological problems we cannot hope for the minute exactness of the chemist, but the chemist has his standard and authorised methods, which he dare not depart from. When a method is being improved or a new one instituted a committee and official referee is appointed, and a thorough test, often extending over years, is given. It seems that such methods would be helpful in bacteriological technique and help to ward aside many criticisms that are at present levelled at us. The Society might add to the committees already at work, and assign more problems to be worked out for the common good.

VII. BUREAU OF EMPLOYMENT

The Chemists Club of New York city has managed an employment agency since 1913 with marked success. I have frequently availed myself of its services with satisfaction to all concerned, and I suggest that our society might organize and run a similar bureau. If this employment bureau were incorporated as a "membership corporation" no person could benefit by the profits but any such accruing would go to the Society. Such an organization, if carefully organized and conservatively run, should be of great service to those in administrative positions, or heads of departments desiring to obtain assistance, not to mention those aspirants to bacteriological fame who desire to place a foot on the first rung of the ladder of employment opportunity. Certain large employers of trained bacteriologists, such as the health departments of large cities, could file their specific needs with the bureau, and these organizations could be kept informed of any men coming on the list who were specially fitted for their particular line of work.

VIII. A BACTERIOLOGICAL MUSEUM

The American Museum of Natural History in New York has maintained a bacteriological museum under the skilled supervision of Prof. C.-E. A. Winslow. This has been a valuable asset to members of the Society, and should have support. Some of the older members may remember the Museum which Kral maintained in Prag, and the fine series of museum specimens and photographs he used to prepare. Kral's collection was very helpful to teachers and investigators, and has now been moved to Vienna.

The Society should have a museum, which should serve as a repository for all type species of organism described in our literature; further, I suggest that part of the work of such a museum would be to keep up the pathogenicity of organisms of economic importance, or those used for teaching. For several years I have sought plant pathogens of known virulence, for class work, but have received organisms devoid of pathogenicity, to the disappointment of the classes, and the possible loss of faith in one's veracity.

If the American Natural History Museum will undertake this work, nothing more need be done by the Society, save to let all members know what services the museum can render. If, however, some measure of support is necessary, I trust that the Society will investigate in what way the interests of its members can best be served, and make proper business arrangements with the Museum.

I suggest also another function for a museum, that of a

IX. BACTERIOLOGICAL CLEARING HOUSE

You are all aware of the functions of a clearing house in a large city, an organization the members of which meet daily and pass through the various cheques and transactions of many banks.

Something similar would be valuable to the bacteriologist. I presume that many of you have had similar experiences to my own. Working on a particular problem, or doing some routine work, such as water or milk analysis, you find occasionally an

organism that attracts your interest by some peculiarity or abnormality. You isolate it, and put it by, with the intention that at some convenient season you will investigate it further, but alas, procrastination is the thief of time; the convenient season does not come, and the organism probably dies of exhaustion, starved by inattention to its material needs.

Now, if we had a clearing house the course would be different. You would say, candidly, I have not the time to work out this organism; it is interesting on account of its morphology or some peculiarity of its culture; I shall send it to the clearing house with a note as to where it was found, and its peculiarities. It is, therefore, duly dispatched, and on arrival at the clearing house, those in charge will read the letter and note the peculiarities, and will say, send this to John Doe, he is interested in this line and is working on this particular group, or investigating this irregularity, etc., or failing a student of this group, the organism might be investigated by those in charge of the clearing house.

This is a function that a well equipped and adequately staffed museum might undertake—possibly the American Museum of Natural History might institute a department charged with such work. If this were possible it would be a great asset to our Society. Failing such an organization, it might be possible to arrange for some distribution through the Journal.

X. A NEW DETERMINATIVE BACTERIOLOGY

Those of us who teach and those who are engaged in general or systematic studies, know the difficulties experienced in determining species.

Frantic search of Chester, Migula, Matzuschita, of monographs here, of periodicals there, often fail to give any information or assistance on the points we desire, and then we realise the inadequate nature of our descriptions and our classifications, for they are numerous.

The Society, through the interest of some of its members, has taken cognizance of the chaos in classification and has adopted certain revisions which are helpful.

The Society also has a chart that has undergone a number of revisions, and which in its present form represents what is considered necessary for a proper description of an organism. These two contributions are excellent, but not enough. We want full descriptions as per Society chart of all known organisms, and we want them properly named and classified according to our latest classification. Here then is a splendid task for the Society, a task seemingly of great magnitude, but with proper organization and coöperation we should be able to surmount all obstacles, and produce a new determinative bacteriology approved by the Society, and all interested in bacteriology. Such a publication from its intrinsic value would find a place in every laboratory and succeeding generations of students and workers would rise up and call us blessed.

We have a membership of a thousand. If each one would pledge himself to give a full description of an organism assigned to him, what a magnificent start it would be. I trust the Society will accept this suggestion and formulate the necessary committees.

XI. TEACHING PROPAGANDA FOR BACTERIOLOGY

A subject, the study of which may not only serve on account of its educational value by enlarging our knowledge of nature and training the powers of observation and judgment, but also because of its sheer practical utility as the servant of medicine, pathology, sanitation, industry, agriculture, and household life; should have a well defined place in all our colleges and universities, and possibly in secondary schools. Yet we find that it is not mentioned in the curriculum of eight of our agricultural colleges. In many medical colleges it takes a subordinate place, and in many institutions having comprehensive courses in botany and zoölogy no course is given in bacteriology. Surely this is not right. Several past presidents of our Society have spoken about the value of the subject from the educational and practical aspects, notably the late Prof. W. T. Sedgwick and Professors Bergey, Marshall and Jordan, and there are excellent references in our literature as to the place the subject ought to

hold in our halls of research and learning. Therefore, all members of this Society should carry on incessantly and enthusiastically an active propaganda for bacteriology, for more bacteriology, and for still more bacteriology until the subject becomes more widespread in its benediction and embraces all classes and all institutions of secondary and higher learning.

A subject, which has achieved so much for the relief of suffering and the prevention of disease and whose effective progress and definite mission can be measured almost daily merits the early attention of the pupil and student.

Let us remember the words of the Greek philosopher, Prodicus, "That which benefits human life is God."

CONCLUSION

In conclusion, let me state that I have made these suggestions in the hope that they will be freely discussed by our members. We have a live Society, we are all interested in a subject that has advanced in the last forty years by leaps and bounds, there is much to investigate and nothing can provide an investigator with quite so pleasant a decoy as the persuasion that his chosen theme is in the nature of virgin soil. Here is a true adventure of the spirit, for he is adding a few sovereign grains of gold to the riches of his science for the enrichment of present life.

> If little labour little are our gaines;
> Man's fortunes are according to his paines.
>
> —*Herrick.*

NOTES ON THE GRAM STAIN WITH DESCRIPTION OF A NEW METHOD[1]

VICTOR BURKE

From the Department of Pathology, Stanford University, San Francisco

Received for publication June 2, 1921

An examination of the literature concerning the Gram method of staining bacteria and of the methods of making the stain in various laboratories reveals the fact that a successful stain, i.e., one giving sharp differentiation between Gram positive and Gram negative organisms, can be obtained by several methods. A comparison of these procedures indicates that in a successful method each step is properly coördinated with and influenced by the other steps in the process. One step can be modified if other steps are also properly modified. This fact has been recognized and made use of in the various processes employed to improve or shorten the process of making the Gram stain.

Of all the various methods advanced none has successfully eliminated the personal factor. A satisfactory stain, especially of pus smears, depends to some extent upon the skill of the operator. Any one procedure has to be more or less modified depending upon the type of smear to be stained. For this reason the untrained assistant can not always be depended upon to produce satisfactory results by following any method so far advanced.

The skill of the operator, aside from his knowledge of the organisms to be examined, depends, upon the proper control of decolorization and the ability to detect any defect in the primary stain used.

[1] Aided by funds from the United States Interdepartmental Social Hygiene Board for Research in the Prevention and Cure of Venereal Diseases.

The control of the decolorizer requires experience. Some of the methods used advocate exposure of the smear to the decolorizer for a definite period of time, other methods recommend exposure to the decolorizer as long as any of the stain comes out. The skilled technician modifies both of these methods as the occasion arises. The time of exposure to the decolorizer must vary with the type of smears to be examined and depends upon the excellence of the violet stain and decolorizer used and upon the nature of the treatment accorded the smear between the removal of the iodine solution and the application of the decolorizer. It has not heretofore been recognized that the extent of the blotting and accompanying drying of the smear after the iodine solution greatly influences the decolorization of the Gram negative organisms. The process of blotting after the iodine constitutes one of the two critical steps in the Gram stain and will be discussed in detail later. Aside from the blotting after the iodine and the control of the decolorizer, the balance of the process can be carried on by an untrained assistant.

No one method so far advanced has been shown to be distinctly superior to all the other methods. The Committee on the Descriptive Chart of the Society of Bacteriologists has not yet selected any one method as the standard method to be used in the study and description of pure cultures. This committee has presented three methods for the consideration and criticism of the members of the Society. (Conn, H. J., 1919; Atkins, K. N., 1920.) While not recommending any one complete method it does recommend that 100 per cent alcohol be used as the decolorizer, that the films be made with distilled water and that the gentian violet, iodine solution and alcohol be drained or blotted off but not washed off of the film.

The different methods so far advanced vary in the making of the solutions, the strength of the solutions and the time periods of exposure. The most important modifications of the method as presented by Gram have resulted from efforts to improve the primary stain by increasing the penetration and intensity of the stain and the permanency of the solution. In most cases increasing the intensity of the stain by the use of mordants such

as anilin oil and phenol results in a loss in the stability of the solution. The stability of the solution has been found to vary with the method of mixing the ingredients together. The original anilin gentian violet as described by Gram may deteriorate in a few days. Sterling's modified solution lasts several months. Other modifications are said to keep indefinitely. Perhaps the most promising of these is the one recently described by Atkins (1920) in which anilin sulphate replaces the anilin water in the primary stain and NaOH is added to the iodine solution.

One of the objections to the use of the Gram stain has been the unsatisfactory or unstable nature of this primary solution. Until the discovery of Jensen (1912) that an aqueous solution of methyl violet gives very satisfactory results it was assumed that a mordant such as anilin oil was a necessary factor in making a good Gram stain. Aqueous solutions of methyl violet are coming into general use in Europe. That this has not occurred in America is due to the difficulty of obtaining methyl violet 6b, as recommended by Jensen and to the fact that the American dyes have given unsatisfactory results. With the production of better domestic dye stuffs or improved methods of using the present products we believe that the favored primary stain will eventually be an aqueous solution which will be stable over a long period of time. This stability, combined with ease of preparation, are factors in its favor.

The present paper describes the results of experiments to compare aqueous solutions of various domestic dye stuffs and their value as substitutes for the anilin gentian violet solution in the Gram staining method.[2] The dyes used gave variable, and in most cases such unsatisfactory results that the experiments were extended to cover an analysis of the different factors determining the Gram reaction in the hope of modifying the staining method in such a way that satisfactory results could be obtained with more of the domestic dye products. The experiments included the determination of the effect of heat and of acid and alkali added to the primary stain on the slide, the effect of

[2] This work was begun by Mrs. Pearl M. Smith and continued by the writer.

washing between the different solutions, the effect of heat on the iodine solution, a comparison of different decolorizers and the effect of water on decolorization. Certain of the experiments have a bearing on the nature of the Gram reaction and the characteristics of the Gram precipitate.

Organisms used. Unless otherwise stated the smears used in these experiments were made from pure cultures of *Staphylococcus aureus; Bacterium typhosum; Neisseria catarrhalis* and *Neisseria gonorrheae;* grown for approximately twenty-four hours on peptic digest agar slants to which 33 per cent hydrocele fluid had been added or Loeffler's blood serum tubes.

Films. The films were made in tap water or physiological salt solution. A few preliminary experiments convinced us that no difference resulted from the making of the films in distilled water, tap water or salt solution. It was assumed that if a difference did result this would be a factor that would have to be taken into consideration in staining pus and body fluids. Mounting the films in an acid or an alkali does influence the results as will be described later.

DYES USED IN PRIMARY STAINING SOLUTION

One per cent aqueous solutions of six different samples of methyl violet, three of gentian violet and one of crystal violet were used and compared. The dye was added to the distilled water, shaken thoroughly, allowed to stand several hours and filtered as used. Such solutions remain stable for a considerable time. Some of our solutions were kept two months and showed no deterioration, in fact there seemed to be some improvement with age.

Results obtained. According to the method of Jensen, satisfactory results can be obtained with aqueous solutions of methyl violet 6b, only when using a strong iodine solution[3] and absolute alcohol as the decolorizer. With our dyes we compared the results obtained with the following decolorizers, 95 per cent alcohol, 100 per cent alcohol, 100 per cent acetone and acetone

[3] One gram of iodine, 2 grams of potassium iodide, 100 cc. of distilled water.

and ether (equal parts), otherwise following the method of Jensen except that safranin was used as a counter stain.

Briefly, Jensen's method is as follows: Air dry, fix with mild heat, cool before flooding with 0.5 per cent methyl violet solution for fifteen to thirty seconds; rinse off methyl violet with iodine solution, flood with fresh iodine solution for thirty to sixty seconds, drain off iodine solution and wash with absolute alcohol until stain ceases to come out of film; counter stain for fifteen to thirty seconds with neutral red made up as follows: 1 gram neutral red, 2 cc. glacial acetic acid, 1000 cc. distilled water.

With 95 per cent alcohol as the decolorizer only one of our dyes gave satisfactory results. Using absolute alcohol four of the dyes, three of the methyl violets and the crystal violet, gave good differentiation. Better results were obtained with acetone or acetone and ether than with absolute alcohol. These results show that all American methyl violet dyes do not give satisfactory results when used according to the method of Jensen. Of the dyes used one of the methyl violet dyes gave distinctly superior results and another distinctly inferior results. The crystal violet dye gave better results than some of the methyl violet and any of the gentian violet dyes used.

The dyes were found to vary in the amount of precipitate formed upon the addition of the iodine solution and the rapidity with which this precipitate went into solution in the decolorizer. The dye giving the poorest results produced a heavy precipitate which went into solution slowly. This required longer exposure to the decolorizer which partially accounts for the poor results. One of the essentials of a good dye should be that the precipitate formed with the iodine solution go into solution in the decolorizer very rapidly.

An attempt to modify the method of Jensen so that satisfactory differentiation can be determined with a larger percentage of American dye products was successful and is given in detail at the end of the article.[4] We will discuss here only certain

[4] The methyl violet dye giving the best results, i.e., resisting decolorization the longest, was a sample furnished by the Will Corporation of Rochester, New York, and submitted to us as their methyl violet No. 3. With the method of

parts of this technique which are deemed worthy of special attention.

EFFECT OF SODIUM BICARBONATE WHEN ADDED TO THE PRIMARY STAIN

The addition of a few drops of a strong solution of sodium bicarbonate to the dye on the slide improves the intensity of the stain in the Gram positive organisms. A few drops of 10 per cent lactic acid produces the opposite result. No attempt was made to determine to what these changes are due. We are possibly dealing with changes in osmotic pressure and subsequent concentration of the dye in the cell or with a change in the size of the molecule of the iodine-dye precipitate, or simply with a heavier precipitate. The sodium bicarbonate tends to precipitate the dye but the acid does not. The sodium bicarbonate and lactic acid do not produce a permanent change in the cell as is readily shown by exposure of the film to either one of the solutions and then changing the reaction by the addition of the other solution. A film so treated will stain as though the first solution had not been used (Burke, 1921).

The effect of the sodium bicarbonate is shown in staining films made from old cultures of Gram positive organisms. Such films stained by the ordinary methods show many organisms which are Gram negative and some which are Gram amphophile or Gram positive. Similar films stained with sodium bicarbonate added to the violet dye will show a larger percentage of Gram positive organisms. Apparently some of the Gram amphophile organisms have absorbed or retained a larger amount of the dye and appear Gram positive. Sodium bicarbonate does not tend to make a naturally Gram negative organism Gram positive. These facts suggest the possibility, not heretofore recognized, that the loss of the Gram positiveness of organisms in old cultures is not entirely due, as formerly assumed, to autolysis and altera-

staining described in this article satisfactory results were obtained with a crystal violet and methyl violet furnished by the National Stain and Reagent Company of Norwood, Ohio; three methyl violet and a gentian violet furnished by the Will Corporation of Rochester, New York; and a methyl violet and gentian violet furnished by the Harmer Laboratories of Philadelphia.

tion of the cell wall but may be due in part to the presence of acid.

The application of these facts in the study of slow growing organisms is obvious. Its value in the examination of pus and body fluids is also evident and has been discussed in a separate paper (Burke, 1921).

The sodium bicarbonate solution may be omitted from the Gram stain if the best dyes are used. We have found that 3 to 8 drops of a 5 per cent solution of sodium bicarbonate solution is usually sufficient to insure good results. If too much sodium bicarbonate solution is added a heavy precipitate forms and there is an almost complete separation of the dye from the water. This should be avoided. A film forms where a drop of strong sodium bicarbonate hits the dye. This disappears as the dye and sodium bicarbonate are thoroughly mixed.

Sodium bicarbonate should not be added to the stock solution of the stain as there results a rapid breaking down of the solution. Whether the sodium bicarbonate is just as effective when added to the iodine solution was not determined.

EFFECT OF WATER ON DECOLORIZATION

The presence of water on the slide to which the decolorizer is added has a marked effect on the rate and extent of decolorization. The rate and degree of decolorization in the presence of water depends to some extent, according to the physical conception of the reaction, upon the action of the water on the cell wall of the Gram positive organisms and upon the fact that the precipitate formed by the dye and the iodine goes into solution in the decolorizer more rapidly if kept moist than when allowed to dry. It follows that in making a stain it is advisable to remove as much water as possible from the slide without allowing the dye precipitate to become dry before adding the decolorizer. Also that after decolorization of the Gram negative organisms is complete water or an aqueous counter stain should not be added to the slide until the decolorizer has evaporated or been removed. The addition of water to the decolorizer decreases its power to take up the dye precipitate into solution and increases its rate of decolorization of the Gram positive organisms. This

is shown by the fact that eighty per cent alcohol decolorizes the Gram negative organisms more slowly and the Gram positive organisms more rapidly than 100 per cent alcohol. If the alcohol is diluted sufficiently both types of organisms decolorize at the same rate. With the addition of more than 50 per cent water the Gram negative organisms do not decolorize over night.

The explanation of these facts upon a physical basis is that (1) the precipitate is more soluble in alcohol or acetone than in water and (2) the water alters the cell wall of the Gram positive organism or reduces the size of the molecules of the dye precipitate so that the dye is more easily washed out by the decolorizer. With the addition of sufficient water to the decolorizer the Gram positive organisms or the molecules of the precipitate are so altered that the dye comes out of the Gram positive organisms as readily as out of the Gram negative organisms. Conversely the elimination of water from the decolorizer and from the cell results in the Gram positive organisms retaining the dye more tenaciously than the Gram negative organisms.

Unfortunately prolonged exposure to a water free decolorizer will remove the dye from the Gram positive organisms and thorough drying of the precipitate delays its solution in the decolorizer. It follows that if we dry the film too thoroughly before adding the decolorizer the rate at which the dye precipitate goes into solution may be decreased to such an extent that before the dye can be washed from the slide and the Gram negative organisms it may, but does not always, begin to come out of the Gram positive organisms. It is not all washed out of Gram positive organisms by acetone in 12 hours. By taking advantage of these facts it is possible by careful blotting of the film before adding the decolorizer to increase the Gram positiveness of the Gram positive organisms without affecting the rate at which the dye precipitate goes into solution in the decolorizer and the decolorization of the Gram negative organisms. Successful staining with some dyes depends upon the skill with which the water is removed from the film. This constitutes perhaps the most critical step in the process of staining by Gram's method. Success with the poorer American dyes depends upon one's ability to properly gauge the effect of the blotting upon the

decolorization of the Gram positive organism and the skilful control of the decolorizer. With the best dyes it is not necessary to pay special attention to this process although the excess water should always be removed by blotting.

There are then, in so far as water is concerned, three facts which should be understood, controlled and utilized in the making of a Gram stain: 1, Water added to the decolorizer increases its power of decolorizing the Gram positive organisms; 2, Water added to the decolorizer slows down the rate at which the dye-iodine precipitate is taken into solution; 3, A dye-iodine precipitate goes into solution before drying much more rapidly than after drying.

Since a small amount of water must be left in the film and the addition of water to the decolorizer affects the results it is inadvisable to use the decolorizer more than once as is sometimes done when decolorization is brought about by placing slides in Coplin jars. Also the decolorizer takes up but a small amount of the precipitate and quickly becomes saturated and then ceases to decolorize the Gram negative organism.

EFFECT OF THE IODINE SOLUTION

The addition of the iodine solution causes a heavy precipitation of the dye. This precipitate is insoluble in water, but readily soluble in alcohol or acetone and has no staining affinity for cells. It is washed out of the Gram negative organisms more rapidly than out of the Gram positive organisms. It is washed out of the Gram negative organisms more rapidly and from the Gram positive organisms less rapidly than the unprecipitated dye. According to the physical conception of the Gram reaction, the molecules of the Gram precipitate are of such a size that in solution in alcohol or acetone and in the absence of water they do not as readily pass through the limiting membranes of Gram positive as of Gram negative organisms. Neide (quoted by Benians, 1912) states that the potency of the Gram reaction depends largely on the strength of the iodine solution and on the length of the period of application. By using a solution containing double the amount of iodine present

in "Lugol's" solution or increasing the period of exposure to "Lugol's" solution he was able to make some Gram negative organisms retain the violet dye. He also claimed that heating the iodine solution on the slide tends to make the Gram negative organisms retain the stain. Benians studied the effect of steaming the iodine solution for five minutes and found that after this treatment of the *Bacterium coli* organisms either intact or crushed resisted decolorization with 100 per cent alcohol for five minutes. He assumes that the heat causes a chemical change in the dye-iodine precipitate in the presence of the bacterial cell substance and that the precipitate ceases to be soluble in alcohol.

If increasing the exposure to, or increasing the strength of, or steaming, the iodine solution causes the Gram negative organisms to retain the dye then these are factors to be controlled in making a Gram stain. However, in our own experiments we were unable to see that increasing the period of application to two hours or doubling the strength of the iodine solution (2–4–100) had any effect on the decolorization of Gram negative organisms. Likewise the steaming of the iodine solution on the slide for five minutes did not effect the decolorization of typhoid organisms. Films so treated decolorized as rapidly as when exposed to the iodine solution for one minute. If, however, the film became dry during the process the Gram negative organisms retained the dye much longer. In our experiments acetone was used as the decolorizer. The former workers used absolute alcohol which may account for the differences in results.

Our experiments convince us that if acetone or acetone and ether is used as the decolorizer and the film is not allowed to dry steaming or prolonged exposure to the iodine solution will not materially affect the decolorization of the Gram negative organism.

VALUE OF DIFFERENT DECOLORIZERS

Alcohol 95 per cent. Commercial 95 per cent alcohol is used in many laboratories as the decolorizing solution. It gives satisfactory results with some of the better dyes but it can not be used with the poorer dyes. As we have already shown the

addition of water to the decolorizer slows down the rate of the decolorization of the Gram negative and increases the rate of decolorization of the Gram positive organisms. Seventy-five per cent alcohol decolorizes the Gram positive almost as rapidly as the Gram negative organisms. Fifty per cent alcohol decolorizes both types of organism at about the same rate.

Alcohol absolute. Absolute alcohol gives better results than 95 per cent alcohol as there is a greater margin of time between the decolorization of the Gram negative and Gram positive organisms. The high cost and difficulty of obtaining absolute alcohol militate against its use.

Alcohol and acetone. The addition of acetone to the alcohol increases the rate of decolorization of the Gram negative organisms and slows down the rate of decolorization of the Gram positive organisms. Therefore a decolorizing solution of alcohol and acetone gives better results than absolute alcohol.

Acetone. Acetone decolorizes the debris on the slide and the Gram negative organisms from 5 to 10 times as fast as absolute alcohol and the Gram positive organisms much more slowly. Acetone does not completely decolorize the Gram positive organisms if they have been stained with a good dye, in 15 hours. With one of the dyes used by us absolute alcohol decolorized *Staphylococcus aureus* as much in fifteen minutes as acetone did over night. The addition of water to the acetone has the same effect on the decolorization of organisms as the addition of water to alcohol. Eighty per cent acetone gave as good results as 95 per cent alcohol.

Acetone and ether.[5] With 100 per cent acetone the decolorization of Gram negative organisms is almost instantaneous. If desired this rapid decolorization can be slowed down by the addition of ether to the acetone. One part of ether to 1–3 parts of acetone serves as a very good decolorizer. As ether costs about the same as acetone there is little or no economic advan-

[5] It is a common laboratory procedure to use a mixture of alcohol and acetone as the decolorizing solution. Lyon (1920) recommends the use of acetone alone as the decolorizer. We have found that a mixture of acetone and ether is just as satisfactory as acetone.

tage in adding ether to the acetone. Short exposure of the Gram positive organisms to the ether does not affect their Gram positiveness.

ADVANTAGES OF ACETONE AS A DECOLORIZER

1. Reduces time required for the decolorizing process.
2. Gives a greater time period between decolorization of Gram negative and Gram positive organisms.
3. Is cheaper and more readily obtained than absolute alcohol.
4. Gives better results with the poorer dyes.
5. Makes a cleaner slide as the dye is more thoroughly extracted from the debris and clusters of bacteria.
6. Gives better results as it decolorizes the Gram positive organisms more slowly than alcohol.
7. Makes possible the use of a stronger dye or a mordant such as phenol because any heavy precipitate on the slide is quickly washed away.

Alcohol has no advantage over acetone as a decolorizer in the Gram process of staining. When these facts become known we believe acetone will come into more general use as a decolorizer. As has been emphasized by Lyon (1920) acetone can take the place of alcohol in the preparation of pathological sections and in the cleaning and drying of pipettes and other glassware.

Counter stain

The choice of a counter stain and the strength of the solution to be used should be determined by a number of factors. With the better violet dyes the choice of a counter stain is relatively unimportant but with the poorer dyes the choice and control of the counter stain largely determines the excellence of the result. Some of the counter stains in general use have a greater tendency than others to mask the violet dye. One has the choice of either, first, shortening the decolorization with the possibility of leaving a trace of the violet dye in the Gram negative organisms and depending upon a strong counter stain or long exposure to cover over any violet dye remaining in the Gram negative organisms or, second, continuing the decolorization until the Gram negative

organisms are thoroughly decolorized, with the possibility of reducing the brilliancy of the dye in the Gram positive organisms and relying upon a weak counter stain not to mask the dye in the Gram positive organisms. The degree of contrast is the same in both cases but I prefer the former method with a strong counter stain like Safranin O which brings out the Gram negative organisms very distinctly and stains them a color which contrasts more sharply with the blackish purple of the Gram positive organisms than that of some other counter stains. I find it easier to make a decision and study the morphology if both types of organisms are heavily stained with distinct colors than if both are more weakly stained with distinct colors or if one type is intensly and the other very faintly stained.

XYLOL

Clearing the stained film in xylol or turpentine improves the definition, thereby making it easier to separate the Gram positive from the Gram negative organisms and to study the morphology. It is of particular value in the examination of pus and mixed cultures. By clearing the organism in this manner instead of depending upon the immersion oil one can more readily determine whether the density of color in some of the questionable organisms is due to a mixture of the violet dye with the counter stain, or to a masking of the violet dye by the counter stain, or to an excessive concentration of the counter stain.

GRAM REACTION

The phenomenon of the Gram reaction has been explained upon both a chemical and a physical basis. The chemical explanation of the reaction is that the dye, iodine and protein of the cell of the Gram positive organisms form a comparatively insoluble compound. The physical explanation is based upon the assumption, supported by some convincing experiments, that the phenomenon depends upon the nature of the cell membrane and the size of the molecules in the dye iodine precipitate, the molecules in solution in the decolorizer being unable to

pass readily through the cell membrane of the Gram positive organisms owing to the size of the pores. It does not come within the scope of this paper to give a critical analysis of the facts bearing on the above theories. For further discussion the reader is referred to a recent paper by Benians (1920) in which the evidence in support of a physical explanation of the Gram reaction is clearly presented.

We wish to describe here the results of attempts to apply to practical staining methods certain of the conceptions presented in Dr. Benians' article. The results obtained have some bearing on the questions involved and may stimulate others to further research along similar lines.

Benians divides bacteria into three groups as regards the Gram reaction: (1) Gram positive organisms into which the dye penetrates and from which the dye-iodine precipitate is not readily washed out by the decolorizer; (2) Gram negative organisms like the gonococcus into which the dye penetrates but from which the dye iodine precipitate is rapidly washed out by the decolorizer; (3) Gram negative organism of the coliform type into which the dye probably does not penetrate and under certain conditions is not even absorbed into the surface of the cell and which are therefore readily decolorized.

According to the physical conception of the Gram reaction the cell membrane of the Gram positive organism does not allow the passage of the compound dye-iodine molecule when in solution in the decolorizer. If the dye-iodine precipitate in solution in the decolorizer can not pass out of the cell due to the character of the cell wall then we are justified in assuming that under similar conditions the dye-iodine precipitate can not pass into the Gram positive cells. We can also reasonably assume that since the dye-iodine precipitate readily passes outward through the wall of Gram negative organisms like the gonococcus it will just as readily pass inward through the cell wall in so far as physical conditions operate. It follows then that if we expose films of staphylococcus and gonococcus to an alcoholic solution of the dye-iodine precipitate the dye-iodine compound should penetrate the gonococci but not the staphylococci. Also since 75 per cent

alcohol decolorizes the Gram positive organisms very rapidly a solution of the Gram precipitate in 75 per cent alcohol should stain the Gram positive organisms almost as rapidly as the gonococcus like organisms. If this dye-iodine compound could be forced to remain in the gonococci we could stain the films with a weak counter stain which would stain the staphylococci and we would have the phenomenon of a reversed Gram stain. The practical application of such a staining method in the examination of pus for gonococci is obvious.

Unfortunately the addition of iodine to the dye saturates its affinities so that an alcohol or acetone solution of the dye-iodine precipitate does not stain cells. By simply exposing films of organisms to the solution we can not determine whether the molecules of the precipitate have penetrated into the gonococci and not into the staphylococci. The staining power of the solution can be materially increased by the addition of alkali but we do not know what affect this has on the size of the molecules. If the molecules are reduced in size they should enter the Gram positive as well as the Gram negative organisms. If the size of the molecular groups is not altered by the addition of the alkali then the gonococci should be penetrated and stained and the staphylococci not penetrated and not stained or only the surface stained. Exposure to a counter stain or weak decolorizer and counter stain should stain the staphylococci and not the gonococci.

ATTEMPT TO REVERSE THE GRAM PHENOMENON

Experiment 1. Films of pure cultures of *Staphylococcus aureus, Neisseria catarrhalis* and *Bacterium typhosum* were made on a slide in physiological salt solution, air dried and fixed by heat. A sufficient amount of iodine solution was added to a quantity of an aqueous solution of methyl violet to cause a maximum precipitation, the precipitate washed to remove excess iodine and dried. The films were flooded with a saturated alcoholic solution of this precipitate. A few drops of a strong mixture of sodium bicarbonate, sodium phosphate and sodium hydroxide were added to the dye on the slide. After an exposure

of 10 minutes or more the slide was dipped in water to remove free dye and lightly counter stained with aqueous Safranin O. Upon examination the staphylococci were Gram negative and the other two types of organisms weakly Gram positive.

This experiment suggests that the dye-iodine precipitate in solution in alcohol with the addition of alkali more readily penetrates the Gram negative than the Gram positive organisms used. The difference noted apparently is not due to a more rapid decolorization of the staphylococci by the water. If the films are examined after washing and before exposure to the Safranin solution the three types of organisms appear to be equally well stained. Since the counter stain more quickly stains the staphylococci than the other two organisms we assume that the staphylococci are less heavily stained by the dye-iodine precipitate or are only surface stained rather than that the Safranin has a greater affinity for the staphylococci.

Staining by this method, as controlled at the present time is entirely inadequate from a practical point of view for distinguishing staphylococci from gonococci in mixed infections. The results obtained are not uniform and the degree of differentiation is not sufficient.

EXPERIMENTS TO DETERMINE WHETHER THE PRIMARY STAIN PENETRATES THE CELL WALL OF TYPHOID LIKE ORGANISMS

Benians' conception of two types of Gram negative organisms as described above is based upon two experiments as follows:

1. If gonococci and *Bacterium coli*, unfixed by heat, are shaken up in weak solution of methyl violet (1 to 40,000) and then centrifuged, the gonococci are thrown down well colored and the dye is cleared out of the solution while the *Bacterium coli* are thrown down uncolored leaving the whole of the dye in solution. If the *Bacterium coli* organisms were boiled or killed at 65° for thirty minutes they absorbed the dye and were thrown down well colored. The *Bacterium coli* organisms exposed to 60°C. for thirty minutes did not absorb the dye. If the unfixed bacilli were suspended in strong solutions they became deeply colored.

2. When films of *Bacterium coli* organisms and *Bacterium coli*

organisms ground in a mortar were placed on the same slide, fixed by heat in the usual way, treated for two minutes with 0.5 aqueous solution of methyl violet and then decolorized with 95 per cent alcohol it was found that the dye was held much more strongly by the ground up debris of the organism than by the intact. According to Benians "This seems to provide almost certain evidence that the dye had never really permeated the intact bacilli, to get into their substance, as it had been able to get into the substance of the bacilli when broken up. The dyes were therefore only absorbed to the exterior of these intact organisms."

We suggest here that the difference in rate of decolorization between the intact organisms and the amorphous material may have been due to a greater saturation and more rapid drying of the amorphous material rather than to an entire lack of penetration of the dye into the interior of the intact cells.

It seemed important to us, from a purely practical consideration of the subject, to determine whether all the members of the large coli-typhoid-dysentery group of organisms resisted penetration of the dye as had been demonstrated for *Bacterium coli* and to such an extent as to permit of differential staining between these organisms and the gonococcus like organisms. Our experiments were based on three assumptions: (1) That if the cell wall of these organisms resisted penetration by the dye it should resist decolorization of the cell if the dye could be gotten into the cell without altering the cell wall and the decolorizer did not affect the cell wall; (2) That if the organisms were stained with methyl violet, then stained with a dye such as Safranin which slowly covered over the methyl violet and then cleared in xylol the cells should not show a violet center and a Safranin periphery unless the violet had penetrated to the interior of the cells; (3) That if the organisms were stained with methyl violet, partially decolorized with acetone and counter stained with Safranin the center of the cells should not appear violet and the margin of the cell Safranin unless the violet had penetrated to the center of the cells.

Experiment 1. Films of *Bacterium typhosum, Staphylococcus aureus* and *Neisseria catarrhalis* were air dried and stained over night in a 0.5 per cent aqueous solution of methyl violet, then exposed to the decolorizing action of water, weak alcohol, 100 per cent alcohol, acetone, and chloroform. In all cases the typhoid organisms decolorized as rapidly or more rapidly than the other organisms.

Experiment 2. The experiment was repeated with carbol-fuchsin as the staining solution; similar results were obtained. Increasing the period of exposure over the usual two minutes period, increased the time required to bring about decolorization. Apparently the increased exposure brought about a greater concentration or greater penetration of the dye in the cell. There is of course the possibility of a chemical change resulting from the long exposure.

Experiment 3. The above experiments were repeated but with the application of an iodine solution in the usual manner. The typhoid and catarrhalis organisms decolorized at about the same rate and much more rapidly than the staphylococci when exposed to acetone.

From these experiments we conclude that after the dye penetrates typhoid organisms the cell wall offers no greater resistance to the removal of the dye than the cell wall of *Neisseria catarrhalis* and *Staphylococcus*. We have not demonstrated that the dye penetrated the cells but since a similar exposure will result in the staining of acid fast organisms we assume that the dye did penetrate. The following experiments favor this assumption.

Experiment 4. Films of *Bacterium typhosum, Neisseria catarrhalis* and *Staphylococcus aureus* were air dried, stained for two to three minutes in a 0.5 per cent aqueous solution of methyl violet, washed in water to remove excess stain, stained lightly with 2 per cent aqueous Safranin O, washed and cleared in xylol for ten minutes. Upon examination the staphylococci almost uniformly showed a safranin colored border with a dark center. The safranin had penetrated part way into the cells and masked the violet dye. The *Neisseria catarrhalis* organism resembled the staphylococci with the exception that the Safranin had not

penetrated so deeply. The typhoid organism showed less penetration of the Safranin than the staphylococci, in this respect resembling *Neisseria catarrhalis*, but an occasional organism showed a very definite violet center with a Safranin margin. The Safranin masked the violet dye in the staphylococci more rapidly than in the other two organisms.

Experiment 5. The experiment was repeated with films fixed by heat in the usual manner. The results were similar to that obtained with the unfixed films.

Experiment 6. The experiment was repeated with unfixed films with the exception that the smears were partially decolorized by a brief exposure to acetone and ether (equal parts) before being stained with the Safranin. Upon examination many of the typhoid organisms showed a very definite purple center surrounded by a Safranin margin. Some of the typhoid organisms showed irregularly spaced blackish granules in the center somewhat resembling a string of cocci and surrounded by a Safranin colored margin. Other of the typhoid organisms appeared uniformly Safranin colored. As far as the eye could determine the violet dye had penetrated to the center of many if not all of the typhoid organisms.

We conclude from these experiments that in ordinary Gram staining the violet dye penetrates through the cell wall of *Bacterium typhosum*. Our experiments failed to demonstrate that the cell membrane of typhoid like organisms offers much if any greater resistance to the dyes than the cell wall of *Neisseria catarrhalis*. The use of weaker dyes might bring out differences not evident when stronger dyes are used. The solution of methyl violet used by us was of the same strength as used by Benians in one of his experiments with *Bacterium coli* in which he apparently demonstrated that the dye did not penetrate to the center of the cell.[6]

[6] With the Gram stain used by us the typhoid organisms decolorized slightly more rapidly than the *Neisseria catarrhalis* organisms but as the latter were distinctly larger the difference in rate of decolorization may have been due to the difference in size.

These experiments were discontinued because they did not give promise of demonstrating a method by which we could stain the colon-typhoid group of organisms differently from the gonococcus like organisms.

A MODIFIED GRAM STAIN

The following method of making a Gram stain with aqueous solutions of dyes and without the addition of mordants to the stock solution of the primary stain gives better results than any other method known to us. With this method satisfactory differentiation between Gram positive and Gram negative organisms can be obtained with many of the domestic gentian violet, methyl violet and crystal violet dyes. With some of the better dye products certain of the steps in the process can be omitted and others modified, but with the poorer dyes strict attention to all of the details given is advisable.

1. Air dry thinly spread film and fix with least amount of heat necessary to kill the organisms and fix them to the slide (A).
2. Flood smear with a 1 per cent aqueous solution of the dye to be used. Mix with the dye on the slide 3 to 8 drops of a 5 per cent solution of sodium bicarbonate, allow to stand two to 3 minutes (B).
3. Flush off the excess stain with the iodine solution[7] and cover with fresh iodine solution and let stand one minute or longer (C).
4. Wash in water as long as described and blot off all free water until surface of film is practically free of water, but do not allow the film to become dry (D).
5. Decolorize with acetone or acetone and ether (1 part ether to 1 to 3 parts acetone) until decolorizer flows from slide practically uncolored. This usually requires less than ten seconds (E).
6. Blot dry. The slide quickly dries without blotting (F).
7. Counter stain for five to ten seconds or longer if desired with a 2 per cent aqueous solution of Safranin O (G).
8. Wash off excess stain by short exposure to water, blot and dry (H).

[7] One gram iodine, 2 grams potassium iodide, 100 cc. distilled water.

Immerse in xylol or turpentine for several minutes or until clear. Examine.

If the first attempt at staining a smear does not give satisfactory results it is advisable to wash off the oil with xylol, wash off the xylol with acetone and restain. It has been our experience that restaining smears gives better results than the original attempt.

A. The film can be made in either distilled or tap water or physiological salt solution.

B. Some workers recommend cooling the slide before flooding with the dye. With some dyes steaming seems to improve the result or shorten the required period of exposure. Passing the slide through the flame until steaming begins and allowing to stand the two minutes is sufficient. Steaming does not cause the Gram negative organism to resist decolorization. Anilin-gentian-violet can be used in place of the aqueous solution if desired. Allowing the stain to dry around the edge makes a dirty slide but does not affect the Gram reaction. The strength of the solution of the dye and the period of exposure can vary somewhat without affecting the result. A saturated instead of a 5 per cent solution of sodium bicarbonate may be used.

C. The excess stain can be blotted off or washed off by a brief exposure to water. The exposure to the water should be as brief as possible as water tends to reduce the amount of dye in the cells. Washing with water has the advantage of giving cleaner slides and effecting a saving of the iodine solution and the decolorizer.

D. The iodine solution can be blotted from the slide but this has the disadvantage of leaving a small amount of iodine on the slide and with the volatilization following the addition of acetone there is some irritation of the exposed mucous membrane of the worker. If for any reason one can not complete the staining process after reaching this stage it is advisable to place the slide in water until the staining can be completed.

E. The decolorizer should be placed upon the slide, allowed to stand for a few seconds and drained off. Then fresh decolorizer allowed to flow over the surface of the slide until it drops off

clear. Proper control of this process will reduce the amount of decolorizer used to a minimum. Placing the decolorizer in a Coplin jar and dipping the slide up and down in it will not give satisfactory results.

F. Drying after the decolorizer is essential as the aqueous counter stain mixing with the decolorizer has an effect on the Gram positive organisms. The smear can now be examined for Gram positive organisms. The oil can then be washed off with xylene, the slide dried and the counter stain applied.

G. Various counter stains can be used but I prefer Safranin O, or neutral red as recommended by Jensen. With dyes giving a poor Gram reaction it is necessary to reduce the counter staining to a minimum.

H. Washing should be sufficient to remove the dye from the surface of the organisms, flushing for a few seconds will suffice.

CONCLUSIONS

1. Steaming the iodine solution on the slide or increasing the period of exposure does not cause the Gram negative organism to resist decolorization.

2. Drying the film after exposure to the iodine solution greatly delays decolorization of the Gram negative organisms.

3. There are two critical steps in the Gram staining method; (a) the removal of the water after the iodine solution and (b) the decolorization. The control of these steps determines to a large extent the amount of differentiation between the Gram positive and Gram negative organisms.

4. The addition of water to the decolorizer, either on the slide or in the bottle, retards the decolorization of the Gram negative organisms and increases the rate of decolorization of the Gram positive organisms.

5. Acetone is superior to absolute alcohol as a decolorizer. It decolorizes the Gram negative organisms and debris on the slide much more rapidly and the Gram positive organisms more slowly than absolute alcohol.

6. Mounting the films in distilled water, tap water or physiological salt solution does not affect the staining reaction. Mount-

ing the films in sodium bicarbonate or lactic acid greatly affects the result.

7. The addition of sodium bicarbonate results in a greater concentration of the methyl violet dye being present in the Gram positive organisms after decolorization and lactic acid brings about the opposite result. The failure of Gram positive organisms in old cultures and in smears from the genital-urinary tract to retain the violet dye may be due in part to the presence of certain acids. These facts suggest the possibility of enhancing the value of gentian violet in selective media and improving dye therapy by the addition of an alkali.

8. All of the colon-typhoid group of organisms do not differ from the gonococcus-catarrhalis group of organisms in their resistance to the penetration of the methyl violet dye used in the Gram stain.

Results of an attempt to reverse the Gram reaction are described.

There is described a modified Gram stain which, without the use of mordants or alcohol, gives very good differentiation when using many of the American dye products.

RECOMMENDATIONS

1. That the Committee on Pure Culture Study of the Society of Bacteriologists instead of attempting to select any one Gram method to be used as the standard method in the study and description of pure cultures designate the control organisms to be used in checking up the stain. Any Gram method which would give sharp differentiation between the two control organisms could be considered a satisfactory Gram stain to be used in the study of pure cultures. The selection of the control organisms will require considerable study as the organisms selected should have the same size and morphology when grown under identical conditions and should require a first class stain to bring out sharp differentiation. The custom of using staphylococcus and typhoid as control organisms is not satisfactory. The control organisms selected could be sent out to the different

laboratories. We believe this method will bring about more uniform results than the selection of any standard Gram method.

2. That the dye manufacturers attempt to improve the dyes used in the Gram stain by the addition of alkali which will not affect the stability of aqueous solutions of the dye.

REFERENCES

ATKINS, K. N. 1920 Jour. Bact., **5**, 321.
BENIANS 1912 Jour. Path. and Bact., **17**, 201.
BENIANS 1920 Jour. Path. and Bact., **23**, 401.
BURKE, 1921 Jour. A. M. A. **77**, 1020.
CONN, H. J., AND OTHERS 1919 Jour. Bact., **4**, 112.
JENSEN 1912 Berl. klin. Wochenschr., **49**, 1663.
LYON 1920 Jour. A. M. A., **75**, 1017.

DISINFECTION STUDIES[1]

THE EFFECTS OF TEMPERATURE AND HYDROGEN ION CONCENTRATION UPON THE VIABILITY OF BACT. COLI AND BACT. TYPHOSUM IN WATER

BARNETT COHEN

Division of Chemistry, Hygienic Laboratory, United States Public Health Service, Washington, D. C.

Received for publication May 21, 1921

Exposure to extremes of temperature and of hydrogen ion concentration produces an accelerated death rate of bacteria. These are typical conditions of ordinary disinfecting procedures. Temperatures and hydrogen ion concentrations in the zones between those which are favorable and those which are distinctly lethal may be expected to produce an adaptation in the organisms or a comparatively slow death rate, knowledge of which may reveal the conduct of bacteria under moderately unfavorable conditions in nature, and add to our understanding of certain phases of disinfection.

The object of this study was to investigate the effects of variations in moderate temperature and moderate hydrogen ion concentrations upon the death rate in water and dilute buffer solutions. The experimental method imposed sub-lethal conditions upon the bacteria in contradistinction to the accelerated death induced by ordinary disinfecting procedures.

It has been found in unbuffered media like distilled water or tap water that the death rates vary coincidently with apparently unimportant shifts in hydrogen ion concentration. When the pH factor is controlled by means of M/500 buffers, the death rates are stabilized so that comparisons become possible. The

[1] Published by permission of the Surgeon General. Thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Yale University.

pH zone of tolerance, or minimum death rate, of *Bact. typhosum* lies between 5.0 and 6.4; that for *Bact. coli* is wider, and centered near absolute neutrality. The effect of sub-lethal factors is to make a period of induction apparent before death proceeds at a logarithmic rate.

The phenomena of growth, maintenance and death of bacteria are fundamentally important to bacteriology, theoretical and applied; and there are conceivably numerous factors that may influence them. Omitting from consideration in this discussion the obviously large part played by the food supply, we recognize certain predominating physical and physico-chemical factors, chief among which are temperature and the concentration of hydrogen ions. In bacterial death, and in disinfection, which may be regarded as a special phase of bacterial death, their rôle may indeed become paramount.

Temperature plays its important part in the life and death processes of bacteria by controlling the active agencies involved. Referring to the death processes Clark and Lubs (1917) have said: ". . . . in cellular destruction temperature is to be considered as an accelerating *condition* . . . . among the active agents concerned the concentration of hydrogen ions may be of great significance." This opinion is amply supported by experimental evidence reaching back to the days of Pasteur.

As an aid to the study of certain phases of biological processes, the effect of temperature has been widely used and has yielded facts of importance. It has been found empirically that temperature augments physical phenomena in arithmetical progression and chemical phenomena in geometric ratio. As a rule the temperature coefficient of velocity of most chemical reactions for a rise of ten degrees is 2 or more; and for physical phenomena it is nearer 1.

Snyder (1908, 1911) cites numerous examples of these two types of temperature coefficient and shows that in most physiological processes the temperature coefficient of chemical reaction velocity applies. Loeb (1908) ingeniously accounts for the much richer animal and plant life of Arctic waters by reasoning that since a temperature decrease of ten degrees increases via-

bility 1000 times, and of twenty degrees, one million times, while the rate of development is reduced one-third to one-ninth, it therefore follows that at 0°C. many more successive generations may exist simultaneously than at 10° or 20°C. Madsen and Streng (1910) found that the conservation of agglutinins was affected in a like manner; and Kanitz (1915) in his monograph on the subject collects many of the data for convenient comparison.

The rôle of temperature in the growth of bacteria has been recognized from the very beginning. Its effects are found alike in those life phases in a bacterial culture characterized by Buchanan (1918) as the logarithmic growth phase, the maximum stationary phase, and the logarithmic death phase. The present experiments extend the application to the period of accelerated death.

Ward (1895) by painstaking bacterioscopic studies worked out a curve showing the relation between the rate of growth of *B. ramosus* and the temperature of its environment. Barber (1908), doing the same thing with *Bact. coli*, found growth at 30°C. increased two to three times over that at 20°C., and Lane-Claypon (1909) confirmed this observation. Slator (1919) demonstrated the same relationship to hold for the growth and maintenance of yeasts.

Houston (1914) showed that as the temperature was decreased the viability of *Bact. coli* and *Bact. typhosum* in water was increased. The crude experiments of Konradi (1904) showed a similar relationship for staphylococci and other organisms; and Livingston (1921) finds this true for hemolytic streptococci. The magnitude of the temperature effect observed by these authors may only be inferred, however, for their data are mainly qualitative in nature. Paul, Birstein and Reuss (1910) give quantitative data upon the viability of staphylococci and find a temperature coefficient of 2 to 3 for a 10° rise. In the process of disinfection we have numerous examples and a mass of exact data that show the general application of this rule. In this connection we need only mention the classic researches of Paul and Krönig (1896), Madsen and Nyman (1907), Paul (1909) and of Chick (1908, 1910, 1912). In the heat sterilization of bac-

terial spores, Bigelow and Esty (1920) find that the time of sterilization is increased ten times for a 10° reduction in sterilizing temperature. The Committee on Standard Methods of Examining Disinfectants of the American Public Health Association in its last report (1918), has made recommendations for the inclusion of the temperature coefficient as one of the three necessary items in the characterization of disinfectants.

As has been stated above, the relation between temperature and the speed of chemical reactions is as yet upon an empirical basis. van't Hoff (1896) attempted a derivation from thermodynamic considerations but obtained no definite solution. Arrhenius (1889) assumed that reacting substances occur in two tautomeric forms, "active" and "passive," and that a certain quantity of heat is involved in activation. He suggests a relation of the form:

$$v_1 = v_0 \cdot e^{\frac{q}{2}\left(\frac{T_1 - T_0}{T_1 \cdot T_0}\right)}$$

for the velocities of reaction, v_0 and v_1, at absolute temperatures T_0 and T_1, the quantity q being the constant of activation. Numerous empirical formulae of the same general form have been suggested from time to time; and that of Snyder (1911) is one that he applied to physiological processes. Tolman (1921) discusses the views that have been held and points to the lack of a real fundamental explanation of the temperature effect in monomolecular reactions. Dushman (1921) and Lewis and McKeown (1921) offer theories of chemical reactivity which appear to be based upon fundamental considerations and include the effect of temperature.

There is an ever-increasing literature upon the effects of hydrogen ion concentration[2] in many biological processes, as a glance at the references quoted by Clark (1920) will show. In the field of bacteriology its importance as a controlling factor is definitely established. This control is exercised in many subtle and unexpected ways—upon the activity of specific

[2] In this work, the Sorensen symbol pH is used synonymously for the hydrogen ion concentration.

enzymes, upon toxin production, upon the dissociation of essential foodstuffs and upon the state of aggregation of cellular protoplasm. Our present meager knowledge permits us usually to see only the end result in growth, metabolism or death.

Cohen and Clark (1919) found that the acid limit for the growth of *Lactobacillus bulgaricus* is not identical with that for fermentation, thus suggesting a possible distinction between bacterial growth and metabolism. In the experiments to be reported in this work there is a hint that we might extend this distinction to include bacterial death.

These factors, temperature and hydrogen ion concentration, have been utilized in the present investigation to study some of the characteristics of that phase of disinfection produced by mild factors, which for want of a better term we have called sub-lethal.

Beginning with the first systematic studies by Koch (1881), who was later followed by Paul and Krönig (1896), Krönig and Paul (1897), Madsen and Nyman (1907), and culminating in the achievements of Chick (1908, 1910, 1912), there has been evolved a theory of disinfection based upon well known principles of physical chemistry. This may be summarized by the dictum of Phelps (1911): "The rate of dying, whether under the influence of heat, cold or chemical poison, is unfailingly found to follow the logarithmic curve of the velocity law, if the temperature be constant." Lee and Gilbert (1918) come to a like conclusion after a critical investigation.

On the other hand there are some like Reichel (1909), Loeb and Northrop (1917), Brooks (1918), Peters (1920) and Smith (1921) who urge that the logarithmic process is only an apparent one, and that careful study of the intimate nature of the disinfection curve will show it to be dependent upon the individual resistance of the organisms. Brooks supports the contention of Loeb and Northrop that bacteria are distributed according to resistance upon a probability curve and that bacteria of low resistance die off first. Since the logarithmic law takes cognizance of the number of organisms without regard to the distribution of resistance among them, the operation of this law among bacteria would, so they claim, be an unnatural process.

To throw some light upon this controversy and at the same time aid in an understanding of the mechanics of the disinfection process, it seemed that a study based upon the following considerations might prove useful. Most previous studies of disinfection were based upon results of the application of an intense disinfecting agent, like heat, heavy metals, acids, alkalis, etc. As a result, the response of the organisms was prompt and the period of their adjustment to the new conditions was so small as to be overlooked. Only in exceptional cases, as the one cited by Chick, where old cultures of *Staphylococcus aureus* were used, was the early period long enough to excite attention; but in that case the cultures used could not be considered as fairly homogeneous. If the conditions causing death of the organism could be toned down in intensity to a degree that might be termed sub-lethal, then there should be an opportunity to observe their adjustment from their manner of response.

The reduction of lethal intensity may be satisfactorily accomplished by maintenance of the organisms at sufficiently low temperatures on the one hand and by the control of the hydrogen ion concentration of their environment on the other.

EXPERIMENTAL

The series of experiments reported and discussed below was planned to give information on the following aspects of the behavior of bacteria as represented by the colon-typhoid organisms.

1. The response of bacteria to the sub-lethal factors (in contradistinction to disinfection as ordinarily understood) of starvation and moderate intensities of hydrogen ion concentration.
2. The effect of moderate temperatures upon the rate of this response.
3. The analysis of the behavior in the light of the physicochemical concept of the disinfection process.

A statistical method of approaching a solution of these questions appears at present to be the only satisfactory one; and briefly stated, the method of study pursued was to expose *Bact. coli* or *Bact. typhosum* to a given solution and follow periodically

the numbers of survivors capable of forming colonies on nutrient agar.

Materials and apparatus

Close attention was paid to obtaining uniformity of conditions throughout the work. Constant temperatures (10°, 20°, and 30°C.) were secured in an electrically heated and controlled, well-stirred air thermostat, with fluctuations probably not greater than 0.5°C. In the experiments conducted at 0°C. the bottles were maintained at the temperature of melting ice.

The plating materials were of the usual kind, consisting of graduated pipettes, petri dishes, dilution bottles and tubes, and nutrient agar.

The glassware was cleaned in the usual manner and sterilized by dry heat at 160°C. for five hours or longer. The dilution bottles were of about 250 cc. capacity, and were filled from an automatic burette with 100 cc. of distilled water. The dilution tubes were filled with 10 cc. of water. Sterilization in the autoclave brought the contents down on the average to 99 cc. and 9 cc. respectively.

The procedure in making distilled water the suspension-fluid or the diluting fluid is at variance with the customary practice of using so-called physiological salt solution or mixtures of this solution with nutrient broth. This variation was made advisedly because preliminary tests had shown quite conclusively that for our purposes distilled water was a satisfactory neutral fluid (cf. Zeug, 1920).

The nutrient agar for plating was made by a uniform method at various times from a single lot of Difco proteose peptone and Liebig's beef extract. It contained per litre:

	grams
Peptone	10
Liebig's beef extract	3
Shred agar	20

The reaction of the medium was adjusted colorimetrically to pH 7.0. Occasional tests of the reaction of the nutrient agar at the time it was actually used for plating showed the hydrogen ion concentration to be uniformly between pH 6.7 and 6.9.

The distilled water of the laboratory, from a gas-heated Stokes automatic still, was used in all cases except where otherwise mentioned. In experiments requiring water of exceptional purity the latter was prepared by double distillation out of acid and alkali in Pyrex glass, with precautions for the exclusion of carbon dioxide.

The tap water used was Potomac River water which had presumably been treated with alum and filtered through sand. Ficker (1898) has shown that an "oligodynamic" property is readily acquired by water which is allowed to remain in contact for any long time with the metallic fixtures in ordinary plumbing. To prevent this occurrence in our water, the latter was allowed to run freely for several hours before being used for experiment. During filtration, a by-pass provided a constant flow of fresh tap water under a head of one meter.

The buffer solutions were prepared with the precautions described by Clark (1920). The only divergence made here was to dilute these buffers to a concentration of M/500. The pH values of these dilutions were then determined colorimetrically. Tests were made to determine the minimal amount of buffer necessary to maintain constant hydrogen ion concentrations under the conditions of these experiments; and it was found that the M/500 concentration of buffer answered this purpose most satisfactorily.

The bottles in which the bacterial viability was studied were of approximately 1000 cc. capacity, of ordinary glass and with ground stoppers. Before use, they were thoroughly cleansed with fresh chromic acid cleaning mixture, rinsed, steamed in an Arnold sterilizer for several hours, and well rinsed with distilled water.

Except for the experiments with double-distilled water, in which cases the containers received an internal coating of purified paraffin, no attempt was made to prevent possible solution of the glass in the contained water. This procedure was followed because in preliminary experiments a comparison of the viability curves from water in Jena glass containers and in the above softer glass bottles showed no appreciable differences. When

precautions were taken during manipulation to avoid unnecessary and excessive heating, the amount of glass constituents dissolved apparently played no significant part in the experiments as conducted.

Paraffin melting at 55°C. was boiled with several changes of distilled water, stirred frequently and finally skimmed off into clean sterile bottles. The latter were then placed in a hot air sterilizer for several hours and later allowed to cool while a uniform coat was deposited internally.

The organisms selected for experimentation were two members of the colon-typhoid group, *Bact. coli* and *Bact. typhosum*. These were chosen both because they are well suited for this type of study; and because they have been extensively studied along related lines so that results obtained here may be readily compared and may add to the continuity of our knowledge.

Both cultures had been grown for a long time upon artificial media but this does not constitute a defect in the present experimental plan. The undoubted acquisition of higher resistance to external influences altered somewhat the degree of the mortality rate by accentuating and magnifying the retardation during the early phases of the process, precisely the condition desired.

Bact. coli. This organism was isolated from a polluted stream in 1916. Its cultural and morphological characters are typical of the *Bact. coli-communis* (sucrose-negative) type. Winslow and Falk (1918, 1920) have utilized this strain in their studies on salt antagonism.

Bact. typhosum. This was a culture of the Rawlings strain (no. 608) obtained from the American Museum of Natural History, New York.

Stock cultures were preserved on nutrient agar at 10°C. At least five daily transplants on agar at 37° were made before a culture was used for experiment.

The procedure in making the tests. The requisite number of bottles were filled with buffer solution or water, allowing an air space of about 100 cc. and the whole sterilized at one time in the autoclave at 15 pounds pressure for ten minutes. Exceptions

to this procedure were made in the case of the double-distilled water and the filtered tap water experiments. The double-distilled water was freshly distilled directly into sterile paraffined bottles, and the filtered tap water was filtered through a Berkefeld (N) porcelain candle into sterile bottles. The bottles were then placed in the constant temperature box at 0°, 10°, 20° and 30°C., respectively. Duplicate bottles were maintained at each temperature, and kept there at least 12 hours to assume their required temperatures before the beginning of the experiment.

Cultures of the organisms to be studied were grown on agar slants at 37° for sixteen to eighteen hours. A platinum loopful of the growth was carefully removed and shaken into 9 cc. of sterile distilled water. Precaution was taken to remove only the bacterial growth and to avoid taking up any of the medium. A homogeneous suspension of the organisms was then obtained by thorough shaking. The tube was allowed to stand for several minutes to permit sedimentation of particles and the supernatant fluid was used to inoculate the bottles.

The bottles, immediately after being inoculated, were thoroughly shaken to distribute the organisms evenly throughout the volume, and samples were taken to determine the bacterial content by removing 1 cc. of the suspension and plating it in suitable decimal dilutions. Duplicate plates were made of each dilution and, usually, there were 3 or 4 dilutions and sometimes more, of a single sample.

After a suitable period of incubation, usually 48 hours at 37°C., the bacterial colonies on the plates were counted. For the organisms maintained at 0°C. it was necessary to incubate for a longer period to permit growth of colonies of adequate size. The plates were counted and the results of duplicate plates averaged and noted as the bacterial count for the particular moment that the sample was taken.

Bottles were removed from their respective temperature surroundings only for minimal intervals, not more than a minute or two, usually. Otherwise, they were maintained, protected from light, at their respective temperatures. Successive samples to determine the bacterial content were taken at regular

intervals, and at each such time the bottles were thoroughly shaken to secure an even distribution of the bacteria.

Samples were also taken regularly for the colorimetric determination of pH and for the analysis of the absorbed gases.

The determination of absorbed gases in the water was carried out as follows: 5 cc. of the sample were transferred quickly to a Van Slyke (1917) gasometric CO_2 apparatus. The contained gases were extracted, CO_2 determined by means of absorption with KOH, the oxygen determined by means of absorption with alkaline pyrogallol and the volume of the residue noted. Preliminary analyses of air and of air-saturated water showed the method to be satisfactory for this purpose.

By these procedures, a study was made of the viability of *Bact. typhosum* and *Bact. coli* at 0°, 10°, 20° and 30°C. in double-distilled water, in tap water and in dilute buffer solutions.

The experimental results

In early experiments, the mortality of the organisms was often variable, and could be attributed possibly to the presence of soluble constituents from the glass containers. It was first suspected that some salt effect perhaps might be responsible. The results of the first four experiments show clearly the marked effect upon the mortality which was coincident with apparently small variations in hydrogen ion concentration.

Behavior in unbuffered solutions. The result of Experiments 1 to 4 may be most conveniently considered under one head. In these experiments *Bact. typhosum* and *Bact. coli* were each exposed to distilled water and to tap water at 0°, 10°, 20° and 30°C. The course of events is graphically presented in the accompanying charts (figs. 1 to 4).

All the figures are plotted with time as abscissae and the logarithms of survivors as ordinates. The resulting curves show the actual *rate* of decline in numbers. That is, the slope of a curve (the graphic equivalent of the velocity coefficient, k) at any point is indicative of the speed of disinfection and is strictly comparable with the slope of any other curve on the same chart. The experimental points are connected by straight lines without effort to draw "smoothed" curves through greatly

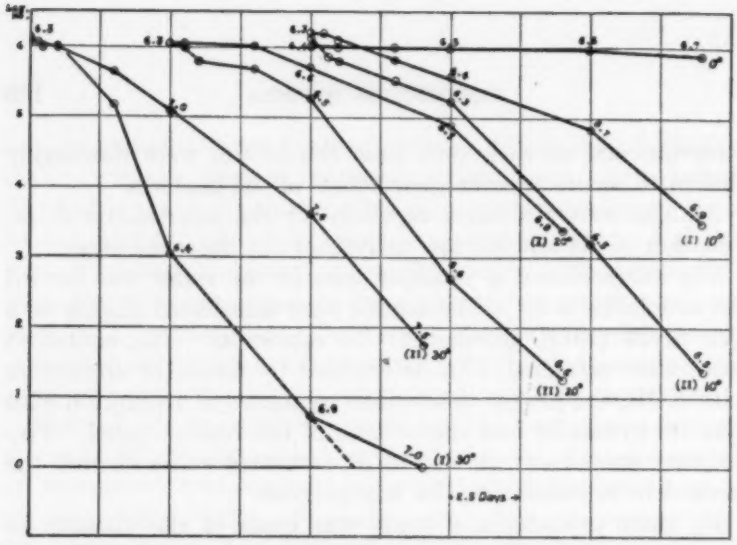


FIG. 1. EXPERIMENT 1. THE DEATH RATE OF BACT. TYPHOSUM IN DOUBLE-DISTILLED WATER AT 0°, 10° 20° 30°C.

Logarithms of numbers of survivors are plotted as ordinates against time intervals as abscissae. Duplicates at any temperature are marked (I) and (II). Observed pH values of the water are noted along the curves.

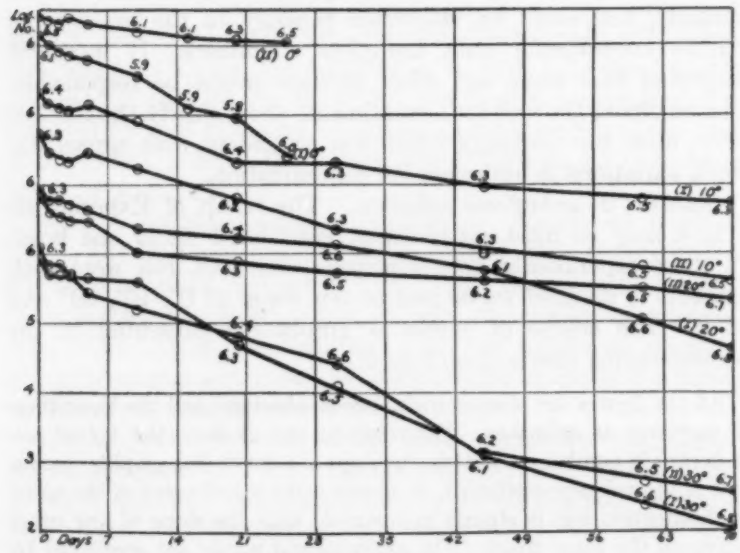


FIG. 2. EXPERIMENT 2. THE DEATH RATE OF BACT. COLI IN DOUBLE-DISTILLED WATER AT 0°, 10°, 20°, 30°C.

Duplicates at any temperature are marked: (I) and (II). Observed pH values of the water are noted along the curves.

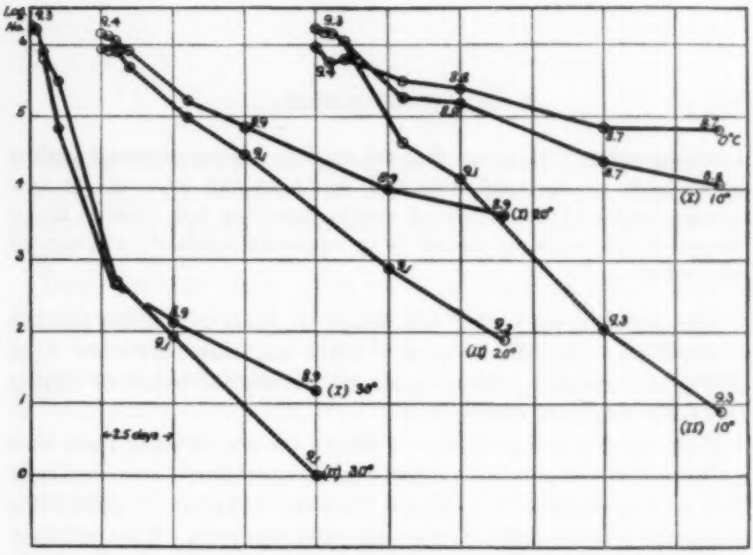


FIG. 3. EXPERIMENT 3. THE DEATH RATE OF BACT. TYPHOSUM IN AUTOCLAVED TAP WATER AT 0°, 10°, 20°, 30°C.

Duplicates at any temperature are marked: (I) and (II). Observed pH values of the water are noted along the curves.

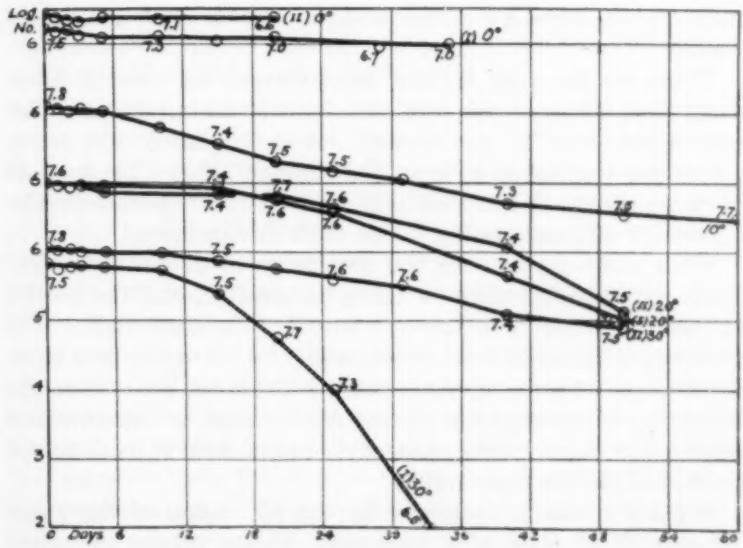


FIG. 4. EXPERIMENT 4. THE DEATH RATE OF BACT. COLI IN BERKEFELD-FILTERED TAP WATER AT 0°, 10°, 20°, 30°C.

Duplicates at any temperature are marked: (I) and (II). Observed pH values of the water are noted along the curves.

deviating points. It is true that the error in the experimental method employed is not inconsiderable and that a smooth curve might adequately represent the course of events occurring but there is also a danger of the eye being misled by an apparent regularity where none may exist.

The well-known higher resistance of *Bact. coli* is here again exemplified. As will be seen further on, this resistance is of utility in helping to throw light upon bacterial behavior during the early stages of disinfection.

Examination of figures 1 to 4 shows certain striking facts that are common alike to both organisms in unbuffered surroundings. The rate of decline in numbers does not always run parallel in duplicate bottles held at the same temperature. This perplexing result is consistently found in these four experiments and inspection at once shows that the divergence is the result of a chance distribution, some curves being generally parallel and others far apart. Of course, this divergence if present increases with the time elapsed, and depends upon the organism; for *Bact. typhosum* being a matter of days, and for *Bact. coli*, one of weeks.

These results were further substantiated six months later, when *Bact. typhosum* was studied in autoclaved tap water. This experiment, no. 12, was carried out in triplicate, and every precaution was taken to treat the samples alike. Yet in spite of these efforts, the mortality in each of the triplicate bottles followed a different course. (See table in Appendix.)

What could account for the divergence of duplicate bottles? They were handled alike as nearly as possible, and the bottles themselves, so far as we knew, were all of the same origin. The dissolved gases could not be responsible, for, as will be seen later, equilibrium with the air was attained within six hours from the beginning of the experiment; and furthermore, in experiment 4 filtered tap water, which presumably retained all of its dissolved gases, yielded the same result.

A possible clue is furnished by the pH values of the water determined at successive intervals. These values are noted along the curves on the charts, and it becomes evident that du-

plicate bottles giving parallel curves show but insignificant deviations in pH from each other. On the other hand, a wide divergence of the curves is coincident with appreciable fluctuations in pH.

These findings apply alike to double-distilled water and to Washington tap water. In the case of the distilled water, which contained a minimum of dissolved salts, the possible contaminants are the atmospheric gases of the laboratory which include, among others, ammonia and sulfur dioxide. The tap water contained in addition to the gases, about 163 parts per million of dissolved mineral matter. Both waters are poorly buffered, with their pH equilibria situated on the steep portion of a curve, so that a trace of strong base or acid will cause a relatively large change in the hydrogen ion concentration. This circumstance makes it difficult to estimate the pH accurately, for the hydrogen electrode cannot be used, and the addition of free indicators, which are themselves strong acids, may vitiate the results. We have, however, made a colorimetric estimation of the pH by utilizing the sodium salts of the Clark and Lubs indicators and comparing the results with those obtained with the free acids.

These pH deviations fall within the physiological range of our organisms, as will be shown later, and it could scarcely be urged, therefore, that these insignificant shifts were the cause of the large variations in mortality. It may however be that the slight pH changes observed were indicative of large and more profound local fluctuations in the unbuffered waters surrounding the bacteria; but it is worthless to pursue these thoughts further for they are purely speculative, and we are ignorant of all the forces operating in the present case. Yet the obvious inference occurs, that the control of the pH with buffers might stabilize a possible factor in the problem and lead to more consistent results. Later experiments are concerned with this aspect of the problem.

Our observations suggest that unbuffered solutions may not be depended upon to retain a constant and uniform hydrogen ion concentration for long, so that we must conclude that results of like experiments in the past may have been vitiated by this factor. Falk (1920) mentions the importance of pH in the study of salt effects upon the viability of bacteria.

It is evident from mere inspection of figures 1 to 4 that effects of temperature, whatever they may have been, were completely screened.

The air-saturation of the suspension fluids

Several considerations made it seem desirable to learn to what extent our water samples were saturated with air gases. The method of analysis used makes no claim to absolute accuracy, but gives a good idea of the actual condition present. Table 1 shows that distilled water, seven days old, was fully aerated at each temperature. To determine how soon saturation would

TABLE 1

Dissolved oxygen and carbon dioxide in distilled water held at different temperatures for seven days

TEMPERATURE	VOLUMES PER CENT			
	Found		Calculated	
	O_2	CO_2	O_2	CO_2
0°	0.98	0.08	0.986	0.072
10°	0.80	0.06	0.796	0.047
20°	0.68	0.03	0.649	0.036
30°	0.53		0.526	

occur under the conditions of our experiments, the following experiment was carried out. Bottles containing 900 cc. distilled water and an air space of 100 cc. were autoclaved, allowed to cool while closely stoppered and then held at 0°, 10°, 20° and 30°C. respectively for twelve hours. The bottles were then thoroughly shaken, and samples removed for gas analysis as indicated in table 2. The results show that within four to six hours the waters were practically saturated with air. (CO_2 determinations are not given because the minute quantities present were within the limits of error of the method employed.)

These observations confirm occasional tests which showed that our bacteria were exposed to an air-saturated environment. As a consequence we may assume that the gas tensions were practically invariant at each temperature throughout these

experiments. That is, the decline in bacterial population at each temperature occurred under practically constant concentration of atmospheric gases. It is interesting to note, however, that at 0°, 10°, 20° and 30°C. the concentrations of oxygen occurred in the ratio of 986:796:649:526. This observation takes on a certain theoretical significance when considered in the light of the findings of Paul, Birstein and Reuss (1910) that dried staphylococci die off at a rate approximately proportional to the square root of the oxygen concentration. The experi-

TABLE 2

*The reabsorption of atmospheric oxygen by autoclaved distilled water**

WATER HELD AT	VOLUMES PER CENT OF OXYGEN				
	Hours after twelve-hour incubation				Calculated for air-saturated water
	0	2	4	8	
0°	0.54	0.88	0.97	0.97	0.986
10°	0.41	0.70	0.79	0.81	0.796
20°	0.25	0.58	0.64	0.66	0.649
30°	0.11	0.47	0.52	0.52	0.526

* The water was autoclaved for ten minutes at 15 pounds, allowed to cool to room temperature and was held in an air thermostat for twelve hours. The water was well shaken before each successive sample was removed for analysis.

ments of Whipple and Mayer (1906) in this connection are interesting. They found that exclusion of oxygen from water containing colon and typhoid bacteria resulted in a rapid decrease in the bacterial population. The contradiction between these observers may perhaps be more apparent than real.

Jacobs (1920) has shown for tadpoles that CO_2 may produce a lethal effect which is accounted for by a specific penetrability. Koser and Skinner (1921) studying the viability of the colon-typhoid organisms found that carbonation of water causes a rapid diminution in numbers. Our experiments throw no light upon this phase of the problem. Carbon dioxide was present in our solutions in presumably constant, small amounts; and its effect should be present to the same degree in all experiments.

The influence of soluble glass constituents

Chemists from the time of Lavoisier have had to consider the question of the solubility of the glass vessels in their work. That a marked influence upon biological experiments may be exerted by soluble constituents from the container has been recognized now and then, but this aspect is yet too often neglected.

Some biological investigators like Beneke (1895) and Ficker (1898) have observed marked responses referable to glass constituents dissolved in the distilled water they used. Molisch (1895) tried to eliminate this source of difficulty by coating his vessels with paraffin. Houston (1914) attempted a comparison of containers of different materials upon the viability of bacteria and obtained some striking qualitative differences. Bigelow and Esty (1920), Esty and Cathcart (1921) and Fabian and Stull (1921) observe differences in heat sterilization referable in part to the solubility of the glass containers, and suggest that the result may be due to pH effects.

Under moderate conditions of temperature, the quantity of material dissolved by water from ordinary laboratory glassware in a short time is comparatively small;[3] and the most obvious results of such solution are the effect upon the pH of the water and the dissociation of other cations and anions. The results of the experiments reported here show that changes in pH of distilled water may often be quite considerable; and tap water, whether sterilized or filtered, has shown itself not very different in behavior from distilled water in this respect.

There are occasional references in the literature to a toxic action exerted by distilled water upon microbes. Laird (1919) found distilled water toxic to staphylococci, which fact he considers, as does Burgess (1920), to be due to plasmolysis and suggests "equilibrated" salt solutions as indifferent suspension media for bacteria. No observations of a similar nature seem to have been reported for bacteria of the colon-typhoid group. Our own experiments demonstrate the prolonged viability of these

[3] It should be noted that our glassware had been in use for some time and had become "aged" to some extent.

organisms in distilled water and tap water, and we must therefore conclude that in this bacterial species plasmolysis is not a very prominent phenomenon.

Winslow and Falk (1918) have demonstrated very interesting salt effects and antagonisms upon *Bact. coli* dying in water; and Holm and Sherman (1921) note similar effects upon growing bacteria. The suggestion of Zeug should be recalled in this connection. It is conceivable that such effects occurred in our experiments but were overshadowed by other more prominent factors. In the light of our experience with the activity of unbuffered suspension media upon bacterial death we must appreciate at once the great experimental difficulties involved in the study of pure salt effects, and the need for caution in their interpretation.

The behavior of Bact. typhosum at different hydrogen ion concentrations

The study of the mortality of bacteria at constant hydrogen ion concentration was made in M/500 solutions of Clark and Lubs' phthalate and phosphate buffers. It was considered desirable to keep the concentration of salt down to a minimum, and this dilution of the buffers was found to be the lowest that would maintain the pH unchanged under our conditions.

Experiment 5 (*Bact. typhosum* at pH 3.8, 5.0, 5.4, 6.4, 7.1, 7.6, 8.7 and 9.5) was performed to determine if constancy of pH would condition a uniformity in the results from duplicate bottles; and to learn the effect of pH at constant temperature (20°C.) upon the viability. Figure 5 shows that the effect of maintaining a constant pH throughout the experiment was to produce a high uniformity in the results from duplicate bottles.[4] As a consequence, duplicate curves, while determined in all cases, are omitted from the rest of the charts for the sake of clarity in presentation.

Reference to figure 5 shows that decline occurs at all hydrogen ion concentrations, and is least at pH 5.0 and 5.4. At pH 3.8,

[4] These are not selected specimens, but include all the tests made.

corresponding roughly to the acidity of N/10,000 HCl, the decline is most acute, and by contrast, at pH 5.0 (equivalent to about N/100,000 HCl), the rate of decline is the smallest. On

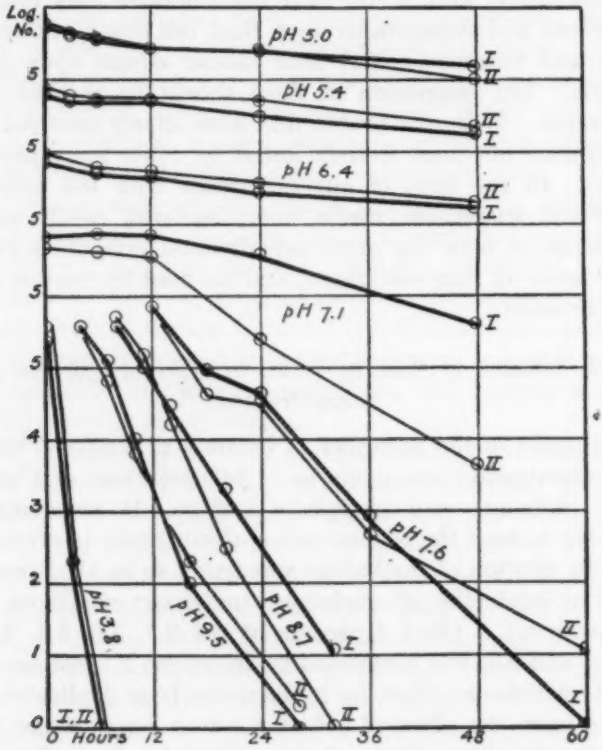


FIG. 5. EXPERIMENT 5. THE DEATH RATE OF BACT. TYPHOSUM IN M/500 BUFFERS AT 20°C.

The curves actually start from nearly the same origin, but have been spread apart to avoid confusion. Note the close parallelism between duplicates at almost all pH values.

the alkaline side, the acceleration in death rate is more gradual. Northrop (1920) studying the acid stability of pepsin found pH 5.0 to favor the greatest stability. He found for this enzyme, however, that increase of acidity did not destroy it so markedly as increase of alkalinity.

Our curves being derived under the same conditions are comparable and their relations may be designated numerically by their slopes, provided the curves are fairly straight lines, as in the present case. This is another way of stating that we are comparing *rates* of decline, or reaction velocities if we prefer to think in terms of the monomolecular reaction. The velocity constants thus derived are given in table 3.

This mode of expressing differences in disinfection has many advantages and has been recommended by Phelps (1911) as one of the logical procedures in the evaluation of disinfectants. Buchanan (1918), Bruett (1919) and Salter (1921) have utilized it in studies of heat sterilization and thermal death points.

TABLE 3

Average velocity constants for the death of Bact. typhosum at different pH values at 20°C.

	pH 3.8	pH 5.0	pH 5.4	pH 6.4	pH 7.1	pH 7.6	pH 8.7	pH 9.5
k	1.055	0.0134	0.0110	0.0138	0.0437	0.1100	0.2134	0.2855
*Relative k	95.5	1.2	1.0	1.5	4.8	10.0	22.4	31.4

* Relative k represents the magnitudes of the various constants compared with that at pH 5.4 taken as unity.

The velocity constants we have thus derived show clearly that there is a zone between pH 5.0 and 6.4 in which *Bact. typhosum* declines in numbers very slowly. A small change in pH toward the more acid side of the zone produces an almost hundred-fold increase in the rapidity of death; while on the alkaline side a similar change in pH increases the death rate only four or five times. In brief, a small increase in the concentration of hydrogen ions at the acid end of the zone of tolerance produces a profound effect, whereas relatively large increases of hydroxyl ions produce a much smaller effect. This coincides with the findings of Cohen and Clark (1919) who called attention to a similar effect upon the *growth* of members of the colon group of bacteria.

This observation is of some importance theoretically, and may help to throw light upon the mechanism involved in the death of

bacteria. For instance, the optimum for growth of *Bact. typhosum* lies between the pH limits 6.2 and 7.2, though it will tolerate greater extremes. Yet here we encounter the significant fact that its optimum for maintenance under conditions of starvation lies between pH 5.0 and 6.4. It would, however, be unwise at present to dwell upon the possibilities in this direction.

Of interest in the same connection is the work by Shohl and Janney (1917) who established the pH limits for the growth of colon and typhoid bacteria in urine. Marsh (1918) found that *Bact. typhosum* is sensitive to the degree of acidity occurring in sour milk; and Beckwith (1920) reports that this organism is capable of surviving in rabbit bile in vivo even when the hydrogen ion concentration is depressed to pH 9.4.

It may be asked how far we are justified in concluding that the control of the pH with buffers conditions uniformity in results between duplicates. Striking graphic proof is given in Experiment 5 in which only one (that at pH 7.1) out of 8 tests failed in this respect. In experiments 6 to 13, the data for which are tabulated at the end of this paper, we find the large majority[5] of duplicates to run closely parallel. That there are a few exceptions is evident but even in these, the average divergence is very much less than in duplicates from deviating unbuffered solutions.

A further test of this fact was made six months later when *Bact. typhosum* was studied in triplicate samples buffered around pH 6.4. In this experiment, no. 13, the technique was especially careful, and the individual bacterial counts are the most accurate we have made, the probable error being below 5 per cent. If the resulting data be plotted against time, it will be found that each of the triplicate samples yields an almost identical curve. We have reason to believe that non-uniformity of results under these conditions is due mainly to laxity in technique.

[5] Of 33 such tests, about 4 may be considered to have shown any considerable divergence between duplicates.

The behavior of Bact. typhosum and Bact. coli at pH 3.5 at different temperatures

Since it became possible to eliminate variability in the results between duplicate bottles by means of control of pH, we could proceed to the study of the effect of temperature upon the death of these organisms.

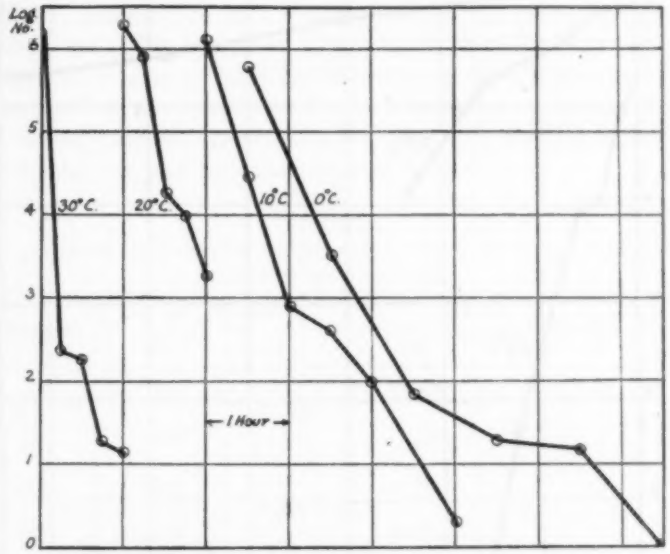


FIG. 6. EXPERIMENT 6. THE DEATH RATE OF BACT. TYPHOSUM IN M/500 PHTHALATE BUFFER, pH 3.5 at 0°, 10°, 20° 30°C.

Closely parallel duplicate curves are omitted

Experiments 6 and 7 were conducted at a comparatively high acidity (pH 3.5) in order to obtain a comparison of the relative rates of dying of these two organisms at 0°, 10°, 20° and 30°C. Such a comparison is not feasible at less intense acidities because of deviations of the disinfection curve from the simple logarithmic form, due to the predominance of certain factors referable to the specific peculiarities of each organism. This will be considered later.

Figures 6 and 7 present the curves of the death rates observed. These experiments are strictly comparable for they were done

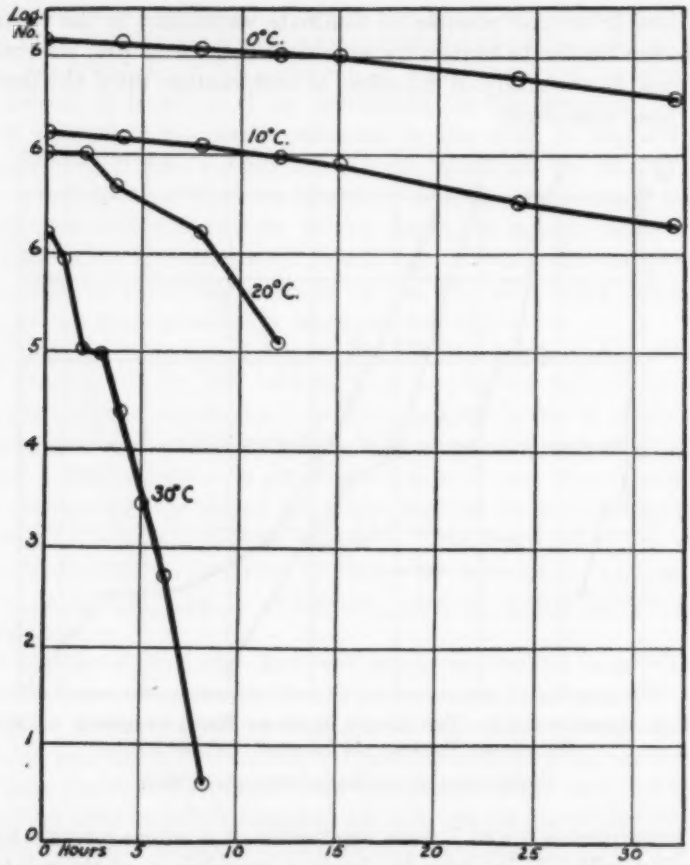


FIG. 7. EXPERIMENT 7. THE DEATH RATE OF BACT. COLI IN M/500 PHTHALATE BUFFER, pH 3.5, at 0°, 10°, 20°, 30°C.

Closely parallel duplicate curves are omitted

at the same time and with identical materials, and the marked difference in resistance of the two organisms is at once apparent. In the case of *Bact. typhosum* (fig. 6) the first portion of each

mortality curve is very nearly a straight line, but at the lower end there is a tendency to flatten out somewhat. In the case of *Bact. coli* (fig. 7) there is a suggestion of this tendency at the beginning of the curve. If we take the average velocity coefficients for comparison, the results may be arranged as in table 4.

We note in the case of *Bact. typhosum* that the increase in the death rate is fairly regular for 10° intervals, being 1.62, 1.53 and 1.77 from 0° to 30°C., while for coli the corresponding increases are 2.12, 4.36 and 3.76. With *Bact. coli* the effect of temperature is to increase the velocity for the 10° intervals from 0° to 30°C. in the ratio of 1 : 2 : 9 : 35 which corresponds closely to the exponential series: $2^0 : 2^1 : 2^3 : 2^5$. Such a relation is not evident in the behavior of *Bact. typhosum*.

TABLE 4

Average velocity coefficients for the death of Bact. typhosum and Bact. coli at pH 5.5 at different temperatures

TEMPERATURE	BACT. TYPHOSUM		BACT. COLI		$\frac{k_1}{k_0}$
	k_1	Q_{10}	k_0	Q_{10}	
0°	1.186		0.0176		67
		1.62		2.12	
10°	1.919		0.0373		51
		1.53		4.36	
20°	2.928		0.1654		18
		1.77		3.76	
30°	5.176		0.6214		8

Q_{10} is the temperature coefficient for the indicated 10° interval.

If the ratio of velocity coefficients be considered a measure of the relative resistance of these organisms, we observe that at 0°C. *Bact. coli* is 67 times as resistant as the typhoid bacillus. As the temperature is increased by ten-degree intervals the relative resistance of *Bact coli* decreases so that at 30°C. it is only eight times that of *Bact. typhosum*.

The behavior of Bact. coli at different hydrogen ion concentrations and temperatures

An inspection of the curves for the death of *Bact. coli* at pH 3.5 (fig. 7) shows that at 30°C. the curve is almost a straight line with a slight suggestion of flattening out at the beginning. At 20°C. this flattening out is more pronounced, and at 10°C. and 0°C., still more so. These deviations cannot be fully accounted for by the probable experimental error, though the process of

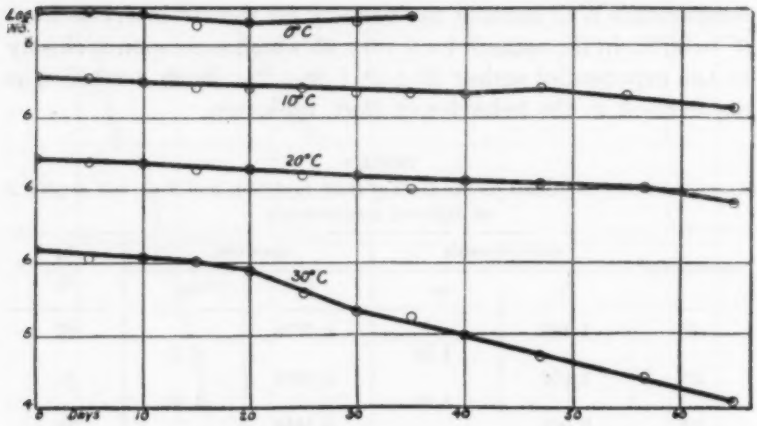


FIG. 8. EXPERIMENT 8. THE DEATH RATE OF BACT. COLI IN M/500 PHOSPHATE BUFFER OF pH 6.1 at 0°, 10°, 20°, 30°C.

Parallel duplicate curves are omitted

drawing 'smoothed' curves through the points may be made to wipe out this deviation. We have found, in experiments not detailed here, that at higher acidities these deviations from the straight-line logarithmic decline are absent so that the resulting curves resemble those found for *Bact. typhosum* in experiment 6.

If we now refer to figures 8, 9 and 10, it will be observed that these deviations are enhanced, especially for the lower temperatures. Such deviations simply mean that as time progresses

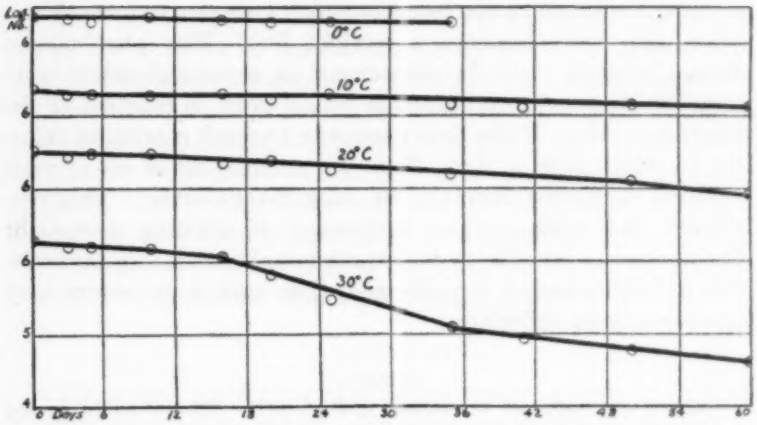


FIG. 9. EXPERIMENT 9. THE DEATH RATE OF BACT. COLI IN M/500 PHOSPHATE BUFFER OF pH 7.1 at 0°, 10°, 20°, 30°C.

Parallel duplicate curves are omitted

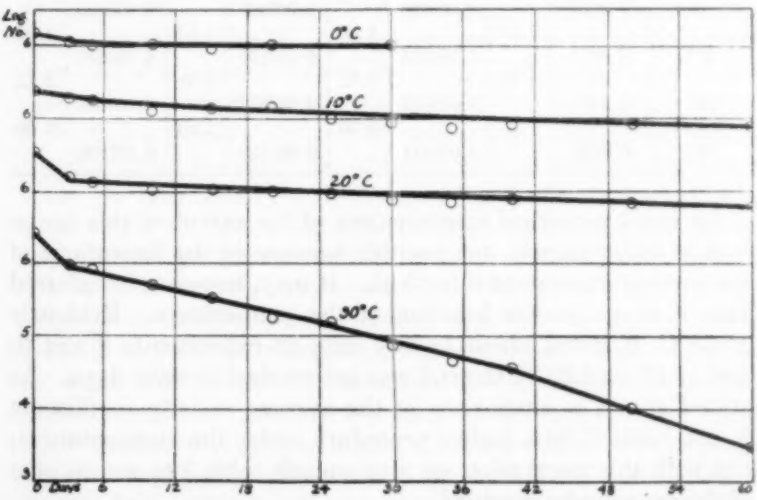


FIG. 10. EXPERIMENT 10. THE DEATH RATE OF BACT. COLI IN M/500 PHOSPHATE BUFFER OF pH 8.0 at 0°, 10°, 20°, 30°C.

The pH remained constant until the fortieth day, but was found to be 7.7 on the sixtieth.

the speed with which the bacteria perish increases to a maximum when the curve becomes a straight line. This phenomenon among bacteria finds its counterpart in certain chemical reactions which possess an induction period prior to reaction at the maximum rate. If the time necessary to reach maximum velocity in death rate is short then the calculation of an average velocity coefficient involves an insignificant error. This represents the mathematical equivalent of drawing a straight line as close as possible to the experimental points. If, however, this induction period is prolonged, then such a procedure may involve serious difficulties.

TABLE 5

Average velocity coefficients of mortality of Bact. coli at different pH values and temperatures

TEMPERATURE	pH 3.5		pH 6.1		pH 7.1		pH 8.0	
	k	Q_{10}	k	Q_{10}	k	Q_{10}	k	Q_{10}
0°	0.0176		0.000206		0.000107		0.000260	
		2.12		1.53		1.62		1.22
10°	0.0373		0.000315		0.000174		0.000314	
		4.34		1.12		2.99		2.12
20°	0.1654		0.000353		0.000520		0.000666	
		3.76		4.56		2.29		3.00
30°	0.6214		0.001611		0.001170		0.001996	

An exact numerical measurement of the extent of this deviation is unfortunately not possible because of the limitations of the present experimental method. It may, however, be inferred that it is an inverse function of the temperature. Evidently at 30°C. it lasted about twenty days in experiments 8 and 9; and at 10° and 20°C. the end was not reached in sixty days. As stated above, a comparison of the average velocity coefficients is not justified as a logical procedure under the circumstances; but with this reservation we may consult table 5 to get an idea of the magnitudes involved.

This table indicates that the pH zone favoring the lowest destruction rate of *Bact. coli* is around absolute neutrality. It appears that this zone covers a greater range of hydrogen ion concentration than does that of *Bact. typhosum*.

We find in these experiments striking examples of that preliminary period in the disinfection process which we have referred to as the period of induction. The bacteria do not begin to die off at the maximum rate, but the mortality increases to the maximum gradually, depending upon the pH and the temperature. The lower the temperature and the less extreme the acidity, the greater is the duration of this period. We have succeeded in this case, apparently, in magnifying that early phase in disinfection which ordinarily is so small as to escape observation. Its significance will be discussed presently.

DISCUSSION

The probable error involved in the method of experimentation leaves much to be desired. Under the most nearly ideal conditions some of the results involve a probable error of only 2 to 3 per cent of the mean values indicated. In others, the probable error amounts to 10 per cent of the mean or more. When the bacterial numbers per unit volume are low, then the probable error increases greatly. These considerations led to the choice of an initial number of one million per cubic centimeter as involving the least manipulative error.

It might perhaps be well to express here a word of caution in regard to a too ready use of the calculated velocity coefficient, k, in the comparison of disinfections under varying conditions. This mode of expressing differences in disinfection has many advantages, provided its use implies no unwarranted assumptions. The general curve of disinfection, plotted logarithmically, is a straight line in the main, but at each end there are characteristic deflections. If these deflections are of small extent then an average velocity constant calculated for the whole of the disinfection period will involve no serious error. If, however, there is a lengthened period of induction before disinfection proceeds at the maximum rate, then obviously, such a basis for the calculation of the velocity constant would involve a large error. The same objection would hold for the condition in which the disinfection curve flattens out toward the end of the process. The latter consideration prompted Phelps (1911) to recommend the

determination of the velocity constant up to the point where the numbers of organisms have been reduced by 50 per cent. To avoid error from the former consideration as well, k should be determined for the middle part of the disinfection curve, say, between the points for 25 and 75 per cent reduction.

The temperature coefficient (Q_{10}) in bacterial viability

We have already mentioned the broad empirical generalization that the temperature coefficient is about 1 for the acceleration of physical processes by a rise of 10° in temperature, and 2 or more for chemical processes. The rule is not a hard and fast one, and its basis is obscure. It is therefore important to recognize that the coefficient is at best a suggestive observation until we possess a better knowledge of the mechanism of the temperature effect.

Obviously, in strictly controlled experiments where only one reaction is allowed to take place, a temperature coefficient so derived is of some value. On the other hand, in biological experiments, the observed effect may be the resultant of an unknown number of independent as well as interdependent reactions, which may be both physical and chemical in nature. A temperature coefficient derived under such circumstances must be of doubtful significance. Changes of temperature may affect consecutive reactions in totally different ways. Mellor (1909) and Osterhout (1917) discuss this aspect of the problem and cite illuminating examples.

With these considerations in mind we may note that the temperature coefficients found in this investigation are in the main those to be ascribed to chemical reactions. This would indicate that somewhere in the series of consecutive processes ending in death, the slowest or limiting reaction velocity was that ascribable to the ordinary chemical reaction. Of more theoretical interest is the finding in experiment 7 that the death rates for *Bact. coli* increased for 10° intervals from 0° to 30°C. in an exponential ratio of the form: $2^0 : 2^1 : 2^2 : 2^5$, a regularity that seems more than accidental. Its interpretation is difficult for the present but we have here a suggestion for a possible method

of getting a closer view, perhaps, of one of the mechanisms involved in bacterial death. In this connection might be mentioned the contribution of Watson (1908) who showed that variation in the rate of disinfection due to change in concentration of the disinfectant could be expressed as an exponential function of the concentration.

Another apparent effect of increasing temperature is to decrease the period of induction prior to the logarithmic rate of death. The evidence is incomplete, but it would seem that the duration of induction varies in inverse proportion to some exponent of the temperature.

The effect of pH upon bacterial viability

We have seen that an uncontrolled hydrogen ion concentration may affect the death rate variably in unbuffered surroundings, so much so as to obscure the effects of wide ranges of temperature. When the pH is controlled by means of dilute buffer solutions the death rates become stabilized. Under the latter conditions there becomes evident a zone of pH in which the death rates of the bacteria are at a minimum. For *Bact. typhosum* this zone lies between pH 5.0 and pH 6.4 and for *Bact. coli* it is wider, with the optimum near absolute neutrality (pH 7.0). These zones may be regarded as optima for tolerance under moderate lethal conditions and when compared with the optima for growth show interesting relations, especially in the case of *Bact. typhosum*. The pH zone for optimum growth of this organism lies between 6.2 and 7.2, while here we have found that the zone for greatest tolerance lies between pH 5.0 and 6.4. Cohen and Clark (1919) showed for the colon-dysentery group that a slight increase in acidity beyond the optimum limit for growth caused a very large effect by preventing growth. The same phenomenon has been found in the present study of response to lethal conditions, where a slight increase in acidity caused a prompt change from maximum tolerance to high mortality.

In practical disinfection the intensity of attack, presumably is so great that such variations in tolerance as we have observed would hardly affect the bacterial death rates. Yet the possibility

remains that if a disinfectant be applied in a medium having a pH favorable to high bacterial tolerance, the result might be appreciably affected. It appears that this tolerance constitutes one element in the general condition termed "resistance." The foregoing observations furnish corroboration of the present well-established principle of determining disinfectant values under accurate control of the hydrogen ion concentration (Wright, 1917).

The laws governing the disinfecting process

We may now turn to a consideration of the process of disinfection and the bearing of our experiments upon the laws governing it. To treat the subject fairly and avoid the possibility of misunderstanding, we shall begin from certain fundamental concepts in physical chemistry. One of them is the assumption of the validity of the mass law. This states that at any moment, the velocity of a chemical reaction is dependent upon the relative masses of the reacting bodies as well as their nature.

If only one substance is undergoing change in a reaction then according to the mass law, the velocity of such change will depend upon the nature of the substance and its amount at any given moment, temperature and other conditions remaining constant. This statement of the law of monomolecular reactions is expressed concisely by the relation

$$V = C \cdot k \qquad (1)$$

in which V represents the velocity of reaction, C, the amount of substance and k, a characteristic factor depending upon the nature of the substance undergoing change.

The velocity may be expressed by $\frac{dx}{dt}$, in which x represents the amount of substance changed in the time t; and if the original amount of the substance be designated by a, then a-x will represent the amount remaining after the time t. We may now write the above equation in the form

$$\frac{dx}{dt} = k\,(a - x) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

This expression on integration becomes

$$k = \frac{1}{t} \log \frac{a}{a - x} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

which is the familiar equation of the velocity of a monomolecular reaction and often spoken of as the logarithmic law.

It is perhaps well to emphasize that this formula represents the *course* of events taking place and makes no pretence of indicating the *mechanism* involved. It indicates the effect of active mass. Although the value of k will be greatly modified by change in the nature and intensities of the forces concerned, the essential *form* of the velocity curve will in no wise be altered since it is fixed by its inherent relation to the numbers of reacting molecules and by this factor only. The factor k may include, by its very definition, many influences. Therefore, to establish experimentally the applicability of the monomolecular law in any given case, all the influences must be kept constant while the effect of mass is being observed. For the present, our knowledge of the mechanism of a monomolecular reaction is nil. We may only guess that it involves complex electronic relations (cf. Tolman (1921) and Dushman (1921)). Note that k in the final expression happens to be designated as the velocity constant since time is inversely related to it. If $\log (a - x)$ be plotted against time in the above equation, the resulting graph will be a straight line.

The above theoretical considerations have therefore led us to the deduction of a logarithmic equation that should hold good under the ideal conditions imposed. When we turn to actual experimental observations on monomolecular chemical reactions it is found that this logarithmic law holds good, according as we are able to maintain the ideal conditions. There may occur deflections from the true logarithmic rate at the beginning and the end of the reaction so that instead of a straight line plot of the results, we get a somewhat s-shaped curve. If conditions

are properly selected, the deflections may be reduced considerably. As a matter of experimental fact, they have never yet been completely eliminated (except possibly for radioactive substances).

These considerations are not held to affect the validity of the monomolecular law. They lead us rather to investigate the disturbing influences which, when discovered and eliminated, furnish further support to the law.

We now possess a fundamental concept of the origin of the monomolecular law as related to chemical reactions and may turn to the phenomena observed in disinfection. If the disinfection process be followed by noting the numbers of surviving bacteria at successive intervals we find that the rate at which disinfection proceeds is in general proportional to the number present. This proportionality is not absolutely true but generally so, and the graph representing the course of the process is more or less a straight line with deflections at both ends. Under appropriate conditions these deflections may be eliminated so that the curve becomes very nearly a straight line. The mortality process as observed by Chick and others, when strong disinfecting agents are used, has been shown conclusively to follow the logarithmic rate, or a slight modification of it.

It is needless to stress the self-evident analogy between the course followed by a monomolecular chemical reaction and the course of the mortality process of bacteria. They represent actual observations.

In this connection must be mentioned the statistical deduction of Yule (1910) based upon the theory of probability, that in a population exposed to a single lethal influence, the rate of death will follow a logarithmic course, and when there are a number of sub-lethal causes, the death rate will be a modification of the logarithmic one.

The above facts have been fully explained by Chick and it would seem superfluous to repeat them were it not for the criticisms of Chick's conclusions by Loeb and Northrop (1917), Brooks (1918) and Smith (1921). These authors urge that individual variations among bacteria may be distributed on a frequency curve such that the mortality rate will proceed *apparently* logarithmically.

Now it would of course be extravagant to say, since the course of disinfection is found to follow the monomolecular law, that therefore a single molecular species is concerned. We can only say that whatever the mechanisms are and whatever the number of consecutive reactions may be, they leave dominant the effect of bacterial concentration. We may say in other words that we are fortunate in having to deal with a phenomenon in which the effect of concentration can be found, and that we can formulate the course of the disinfection process in terms of an equation expressing the relation of concentration to the course of the process. We are, aware, however, of some of the factors that affect the course of the reaction. These in the case of chemical reactions, are enumerated and discussed by Mellor (1909). One of them, the effect of successive intermediate reactions, has been applied by Osterhout (1917) in an interesting manner to biological phenomena, and by Winslow and Falk (1920) to controvert the notion that disinfection is due to a distribution of variable resistances.

We have already indicated that even in the case of a simple chemical reaction known to involve only one molecular species, the mechanisms of disintegration are unknown, or at least the subject of dispute. The monomolecular law in such cases shows only the course of the reaction as it is related to the concentration of reacting bodies.

The analogy in the two cases should be plain. In neither case does the monomolecular law tell us anything about the mechanisms concerned. In both cases the monomolecular law formulates the relation between concentration and the course of the process.

If then we say that the course of disinfection is determined by the distribution of varying resistances we add nothing to the formulation of the experimental facts. We could just as well say that the course of a monomolecular chemical reaction is determined by the frequency distribution of resistance among the individual molecules.

In the criticism by Loeb and Northrop, by Brooks and by Smith use is made of the fact that at the beginning and end of the dis-

infection curve there are deviations from the monomolecular rate. This is emphasized as evidence that the monomolecular rate at the middle portion of the curve is more apparent than real. However, with cultures having presumably the same distribution of resistances we have found that the shape of the beginning and end of the curve may be modified at will by changing the exterior conditions.

Consequently, what force do these objections to Chick's theory possess? The chance distribution of variable resistances assumed for bacteria is paralleled by the chance distribution of variable energy quotas in the molecules of a substance. Furthermore, the logarithmic rate observed, whether in chemical phenomena or in disinfection, is actually a statistical resultant of like significance in both.[6] From such considerations we must conclude that the criticisms of the monomolecular theory of the disinfection process are of no fundamental force for they reduce to a matter of definitions only.

It appears that Chick's contribution has a larger significance than merely the application to a special phase of bacterial existence. Yule's statistical deduction, the experimental findings of Loeb and Northrop regarding the viability of *Drosophila*, those of Brooks regarding hemolysis of red blood cells and a number of others of like import, all indicate the operation of a general principle.

CONCLUSIONS

1. The mortality at constant temperature of bacteria in unbuffered media like distilled or tap water is variable and coincident with apparently insignificant pH variations. Controlling the pH by means of M/500 buffer solutions stabilizes this variability.

2. Subjecting organisms of the colon-typhoid group to mild lethal conditions under moderate temperatures and hydrogen ion concentrations tends to magnify the induction period prior

[6] It must be remembered that the logarithmic curve merely integrates the results of all factors. It is a statistical summation and gives no information regarding the forces at play.

to mortality at the maximum or logarithmic rate. This provides an opportunity for studying the early response of the organism to the disinfection process.

3. The period of induction is decreased by higher acidity and by higher temperature. It appears to have a duration inversely proportional to some exponent of the temperature. It is analogous to the induction period occurring in chemical reactions.

4. At constant pH, the relative resistance of *Bact. coli* to *Bact. typhosum* decreases with rise in temperature from 0° : 10° : 20° : 30° in the ratio of 67 : 51 : 18 : 8.

5. At 20°C. *Bact. typhosum* possesses the greatest tolerance within a narrow zone of hydrogen ion concentration delimited by pH 5.0 and 6.4. A slight increase in acidity beyond the zone results in conditions of maximum mortality. For *Bact. coli* the zone is wider and centered about absolute neutrality. Cohen and Clark (1919) found that the pH optima for growth and fermentation of bacteria may be different. It is now shown that the optimum for tolerance may also be distinct.

6. The mortality of bacteria whether by strong disinfectants or by milder agents follows the laws of logarithmic decline. It is shown that the course of the disinfection process can be expressed by mathematical relations comparable to those used in dealing with monomolecular chemical reactions.

I desire to express my cordial appreciation for important criticism and suggestions to Drs. C.-E. A. Winslow, E. Elvove, S. C. Brooks and especially to Dr. W. Mansfield Clark.

REFERENCES

American Public Health Association, Report of Committee on Standard Methods of Examining Disinfectants. 1918. Am. Jour. Pub. Health, **8**, 506.

Arrhenius, S. 1889 The reaction velocity of the inversion of sucrose by acids. Zsch. f. physik. Chem., **4**, 226.

Arrhenius, S. 1915 Quantitative laws in biological chemistry. London.

Barber, M. A. 1908 The rate of multiplication of B. coli at different temperatures. Jour. Inf. Dis., **5**, 379.

Beckwith, T. D. 1920 The viability of B. typhosus in alkaline bile in vivo. Proc. Soc. Exp. Biol. Med., **18**, 36.

Beneke, W. 1895 Die zur Ernährung der Schimmelpilze nothwendigen Metalle. Jahrb. f. wissensch. Botanik, **28**, 487.

BIGELOW, W. D., and ESTY, J. R. 1920 The thermal death point in relation to time of typical thermophilic organisms. Jour. Inf. Dis., **27**, 602.

BROOKS, S. C. 1918 A theory of the mechanism of disinfection, hemolysis, and similar processes. Jour. Gen. Physiol., **1**, 61.

BRUETT, E. M. 1919 Utility of blanching in food canning. Effect of cold shock upon bacterial death rates. J. Ind. Eng. Chem., **11**, 37.

BUCHANAN, R. E. 1918 Life phases in a bacterial culture. Jour. Inf. Dis., **23**, 109.

BUCHANAN, R. E., THOMPSON, C. E., ORR, P. F., and BRUETT, E. M. 1918 Notes on conditions which influence thermal death points. Abs. Bact., **2**, 5.

BURGESS, K. E. 1920 The toxicity towards Staphylococcus of dilute phenol solutions containing sodium benzoate. Jour. Phys. Chem., **24**, 738.

BUSHNELL, L. D. 1918 The influence of cold shock in the sterilization of canned goods. Jour. Ind. Eng. Chem., **10**, 432.

CHICK, H. 1908 An investigation of the laws of disinfection. Jour. Hyg., **8**, 92.

CHICK, H. 1910 The process of disinfection by chemical agencies and hot water. Jour. Hyg., **10**, 237.

CHICK, H. 1912 The factors conditioning the velocity of disinfection. Orig. Communications, 8th Internat. Cong. Appl. Chem., **26**, 167.

CHICK, H. 1912 The bactericidal properties of blood serum. Jour. Hyg., **12**, 414

CLARK, W. M. 1920 The determination of hydrogen ions. Baltimore.

CLARK, W. M., and LUBS, H. A. 1916 Hydrogen electrode potentials of phthalate, phosphate and borate buffer mixtures. J. Biol. Chem., **25**, 479.

COHEN, B., and CLARK, W. M. 1919 The growth of certain bacteria in media of different hydrogen ion concentrations. Jour. Bact., **4**, 409.

DUSHMAN, S. 1921 A theory of chemical reactivity. Calculation of rates of reactions and equilibrium constants. J. Am. Chem. Soc., **43**, 397.

ESTY, J. R., and CATHCART, P. H. 1921 The change in pH of various mediums during heating in soft and Pyrex glass. Abs. Bact., **5**, 6.

FABIAN, F. W., AND STULL, R. C. 1921 A study of the hydrogen-ion concentration of different kinds of glassware when sterilized with buffered and non-buffered solutions. Abs. Bact., **5**, 210.

FALK, I. S. 1920 Studies on salt action. III. The effect of hydrogen-ion concentration upon salt action. Proc. Soc. Exp. Biol. Med., **17**, 210.

FICKER, M. 1898 Ueber Lebensdauer und Absterben von pathogenen Keimen. Zsch. f. Hyg., **29**, 1.

HOLM, G. E. AND SHERMAN, J. M. 1921 Salt effects in bacterial growth. Abs. Bact., **5**, 6.

HOUSTON, A. C. 1908 The vitality of B. typhi in water. 1st Research Report, Metr. Water Board, London.

HOUSTON, A. C. 1909 The vitality of B. coli in water. 3d Research Report, Metr. Water Board, London.

HOUSTON, A. C. 1911 The vitality of B. typhi in water. 7th Research Report, Metr. Water Board, London.

HOUSTON, A. C. 1914 The effect of temperature on the vitality of B. coli and B. typhi in water. 10th Research Report, Metr. Water Board, London.

JACOBS, M. H. 1920 To what extent are the physiological effects of carbon dioxide due to hydrogen ions? Am. Jour. Physiol., **51**, 321.

KANITZ, A. 1915 Die Biochemie in Einzeldarstellungen. Heft I. Temperatur in Lebensvorgänge. Berlin.

KOCH, R. 1881 Ueber Desinfektion. Mitt. a. d. kais. Gesundh., **1**, 1.

KONRÁDI, D. 1904 Ueber die Lebensdauer pathogener Bakterien im Wasser. Centralbl. Bakt., I. Abt., Orig., **36**, 203.

KOSER, S. A., AND SKINNER, W. W. 1921 Viability of the colon-typhoid group in carbonated water and carbonated beverages. Abs. Bact., **5**, 12.

KRÖNIG, B., AND PAUL, T. 1897 Die chemischen Grundlagen der Lehre von der Giftwirkung und Desinfektion. Zsch. f. Hyg., **25**, 1.

LAIRD, J. S. 1920 The chemical potential of phenol in solutions containing salts; and the toxicity of these towards anthrax and staphylococci. Jour. Phys. Chem., **24**, 664.

LANE-CLAYPON, J. 1909 The multiplication of bacteria and the influence of temperature and some other conditions thereon. J. Hyg., **9**, 239.

LEE, R. E., AND GILBERT, C. A. 1918 On the application of the mass law to the process of disinfection. Jour. Phys. Chem., **22**, 348.

LEWIS, W. C. M., AND MCKEOWN, A. 1921 The radiation theory of thermal reactions. Jour. Am. Chem. Soc., **43**, 1288.

LIVINGSTON, G. S. 1921 The vitality and viability of hemolytic streptococci in water. Am. Jour. Hyg., **1**, 239.

LOEB, J. 1906 The dynamics of living matter. New York.

LOEB, J. 1908 Ueber den Temperaturkoeffizienten für die Lebensdauer kaltblütiger Thiere und über die Ursache des natürlichen Todes. Pflügers Arch., **124**, 411.

LOEB, J., AND NORTHROP, J. H. 1917 The influence of food and temperature upon the duration of life. Jour. Biol. Chem., **32**, 103.

MARSH, P. 1918 The survival of typhoid bacilli in sour milk. Am. Jour. Public Health, **8**, 590.

MADSEN, T., AND NYMAN, M. 1907 Zur Theorie der Desinfektion. Zsch. f. Hyg., **57**, 388.

MADSEN, T., AND STRENG, O. 1910 Einfluss der Temperatur auf den Zerfall der Antikörper. Zsch. f. physik. Chem., **70**, 263.

MELLOR, J. W. 1909 Chemical statics and dynamics. London.

NORTHROP, J. H. 1920 The influence of hydrogen ion concentration on the inactivation of pepsin solutions. Jour. Gen. Physiol., **2**, 465.

OSTERHOUT, W. J. V. 1917 Some aspects of the temperature coefficients of life processes. Jour. Biol. Chem., **32**, 23.

PAUL, T. 1909 Der chemische Reaktionsverlauf beim Absterben trockener Bakterien bei niederer Temperaturen. Biochem. Zsch., **18**, 1.

PAUL, T., BIRSTEIN, G., AND REUSS, A. 1910 Beitrag zur Kinetik des Absterbens der Bakterien in Sauerstoff verschiedener Konzentration und bei verschiedenen Temperaturen. Biochem. Zsch., **25**, 367.

PAUL, T., AND KRÖNIG, B. 1896 Ueber das Verhalten der Bakterien zu chemischen Reagenzien. Zsch. physik. Chem., **21**, 414.

PAUL, T., AND PRALL, F. 1907 Die Wertbestimmung von Desinfektionsmitteln mit Staphylokokken, die bei der Temperatur der flüssigen Luft aufbewahrt wurden. Arb. a. d. k. Gesundh., **26**, 424.

PETERS, R. A. 1920 Variations in the resistance of protozoan organisms to toxic agents. Jour. Physiol., **54**, 260.

PHELPS, E. B. 1911 The application of certain laws of physical chemistry in the standardization of disinfectants. Jour. Inf. Dis., **8**, 27.

REICHEL, H. 1909 Zur Theorie der Desinfektion. Biochem. Zsch., **22**, 152.

SALTER, R. C. 1921 A comparative study of hemolytic streptococci from milk and from human lesions. Am. Jour. Hyg., **1**, 154.

SHOHL, A. T. AND JANNEY, J. H. 1917 The growth of Bacillus coli in urine at varying hydrogen ion concentrations. Jour. Urol., **1**, 211.

SLATOR, A. 1919 Yeast growth and the alcoholic fermentation of living yeasts. Jour. Soc. Chem. Ind., **38**, 391.

SMITH, J. HENDERSON. 1921 The killing of Botrytis spores by phenol. Ann. Appl. Biol., **8**, 27.

SNYDER, C. D. 1908 A comparative study of the temperature coefficients of the velocities of various physiological actions. Am. Jour. Physiol., **22**, 309.

SNYDER, C. D. 1911 On the meaning of the variation in the magnitude of the temperature coefficients of physiological processes. Am. Jour. Physiol., **28**, 167.

SNYDER, C. D. 1911 An interpolation formula used in calculating temperature coefficients for velocity of vital activities. Science, N. S., **34**, 414.

TOLMAN, R. C. 1921 Note on the theory of monomolecular reactions. Jour. Am. Chem. Soc., **43**, 269.

VAN SLYKE, D. D. 1917 A method for the determination of carbon dioxide and carbonates in solution. Jour. Biol. Chem., **30**, 347.

VAN'T HOFF, J. H. 1896 Studies in chemical dynamics. Translated by Thomas Ewan. Easton, Pa.

WARD, A. M. 1895 On the biology of Bacillus ramosus (Fraenkel), a schizomycete on the River Thames. Proc. Roy. Soc., **58**, 265.

WATSON, H. E. 1908 A note on the variation of the rate of disinfection with change in concentration of the disinfectant. Jour. Hyg., **8**, 536.

WHIPPLE, G. C., AND MAYER, A. 1906 On the relation between oxygen in water and the longevity of the typhoid bacillus. J. Inf. Dis., Supplement no. 2, 75.

WINSLOW, C.-E. A., AND FALK, I. S. 1918 Studies on salt action, I. Effect of Ca and Na salts upon the viability of the colon bacillus in water. Proc. Soc. Exp. Biol. Med., **15**, 67.

WINSLOW, C.-E. A., AND FALK, I. S. 1920 A contribution to the mechanism of disinfection. Abs. Bact., **4**, 2.

WRIGHT, J. H. 1917 The importance of uniform culture media in the bacteriological examination of disinfectants. J. Bact., **2**, 315.

YULE, G. U. 1910 On the distribution of deaths with age when the causes of death act cumulatively and similar frequency distributions. Jour. Royal Stat. Soc., **73**, 26.

ZEUG, M. 1920 Aequilibrierte Salzlösungen als indifferente Suspensionsflüssigkeiten für Bakterien. Arch. f. Hyg., **89**, 175.

APPENDIX

In the following pages will be found, in tabular form, the detailed experimental results. Most of them have been presented in graphic form in the charts.

The values tabulated are the Briggsian logarithms of the numbers of survivors per cubic centimeter of water or buffer solution. Observations of the pH of the suspending fluid are noted wherever made.

Experiment 1. Mortality of Bact. typhosum in double-distilled water in paraffin-lined bottles at different temperatures

TIME	0°C.	10°C.		20°C.		30°C.	
	I	I	II	I	II	I	II
hours							
0	6.0133	6.2122	6.1399	6.0294	6.0569	6.1335	6.0969
pH	6.4	6.3	6.2	6.4	6.2	6.3	6.3
6	6.0038	6.1584	5.9085	6.0128	5.9754	6.0645	6.0645
pH	6.3	6.1	6.2	6.1	6.1	6.3	6.3
12	6.0112	6.1959	5.9004	6.0453	5.9814	5.9978	6.0086
24	6.0008	6.1038	5.8000		5.7882	6.0128	5.9685
72	5.9981	5.8209	5.5366	6.0064	5.7059	5.1847	5.6609
120	5.9780	5.5933	5.2304	5.7251	5.2672	3.0492	5.0934
pH	6.5	6.5	6.4	6.6	6.5	6.4	7.0
240	5.9664	4.8382	3.2014	4.8949	2.7076	0.7782	3.6201
pH	6.6	6.7	6.5	6.7	6.6	6.9	7.4
336	5.8782	3.4900	1.3802	3.3962	1.2553	Sterile	1.7634
	6.7	6.9	6.5+	6.8	6.6	7.0	7.5

Experiment 2. Mortality of Bact. coli in double-distilled water contained in paraffin-lined bottles at different temperatures

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
hours								
0	6.1430	6.4116	6.3351	5.7340	5.9619	5.8325	6.1103	5.9474
pH	6.1	6.5	6.4	6.3	6.3	6.1	6.3	6.2
24	6.0000	6.3617	6.1761	5.4425	5.7782	5.6096	5.8439	5.7076
48	5.9074	6.3234	6.1038	5.3617	5.7938	5.5366	5.8506	5.7235
72	5.8470	6.3962	6.0679	5.3522	5.7160	5.4871	5.7059	5.6794
120	5.8075	6.3040	6.1504	5.4472	5.6314	5.4848	5.4298	5.6551
240	5.5575	6.1895	5.9143	5.2227	5.3560	5.0170	5.1818	5.5877
pH	5.9	6.1						
360	5.1106	6.1096						
480	4.9410	6.0852	5.3181	4.8109	5.2330	4.8882	4.8055	4.6911
pH	5.8	6.3	6.4	6.2	6.4	6.3	6.4	6.3
600	4.3766	6.0311						
pH	6.0	6.5						
720			5.3075	4.3424	5.1367	4.7177	4.4082	4.1004
pH		6.8	6.3	6.3	6.5	6.5	6.6	6.5
1080			4.9455	3.9768	4.7435	4.5988	3.0899	3.1399
pH			6.3	6.3	6.4	6.3	6.1	6.2
1464			4.7931	3.8261	4.0607	4.4843	2.3909	2.7243
pH			6.5	6.5	6.7	6.7	6.6	6.5
1680			4.7435	3.5786	3.6263	4.3345	2.0000	2.5611
pH			6.3	6.5	6.7	6.6	6.8	6.7

Experiment 3. Mortality of Bact. typhosum in autoclaved tap water at different temperatures

TIME	0°C.	10°C.		20°C.		30°C.	
	I	I	II	I	II	I	II
hours							
0	5.9468	6.1875	6.2279	6.1492	5.8779	6.2648	6.2330
pH	9.4	9.3	9.4	9.4		9.3	
6		6.1399	6.1553	6.1206	5.8603	6.1732	6.2279
pH		9.1	9.2	9.2	9.2	9.3	9.3
12	5.7374	6.1303	6.1523	6.0494	5.8470	5.8149	5.8938
24	5.8000	6.0170	6.0294	5.9009	5.6821	4.7803	5.4871
72	5.4813	5.2504	4.6444	5.2175	4.9836	2.6551	2.6875
120	5.4009	5.1818	4.1523	4.8414	4.4728	2.1271	1.9542
pH	8.8	8.8	9.1	8.9	9.1	8.9	9.1
240	5.3372	4.3784	2.0607	3.9671	2.9128	1.2014	0.0000
pH	8.7	8.7	9.3	8.9	9.1	8.9	9.1
336	5.3195	4.0569	0.9031	3.6385	1.9031		
pH	8.7	8.8	9.3	8.9	9.2		

Experiment 4. The mortality of Bact. coli at different temperatures in Berkefeld-filtered tap water

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
hours								
0	6.2156	6.4267	6.0682	6.1345	6.0187	6.0278	5.8319	6.0682
pH	7.5	7.5	7.8	7.8	7.6	7.6	7.5	7.8
24	6.1697	6.4123	6.0800	6.0738	5.9557	5.9863	5.7513	6.0203
48	6.1967	6.3399	5.9782	6.0286	5.9494	5.9474	5.7917	6.0294
72	6.1242	6.3238	6.0064	6.0838	5.9727	5.9978	5.8082	5.9863
120	6.0856	6.3713	6.0382	6.0569	5.9600	5.8904	5.7745	5.9948
240	6.1196	6.3577		5.8028			5.7185	5.9868
pH	7.3	7.1						
360	6.0294			5.5611	5.9360	5.8537	5.4014	5.8692
pH				7.4	7.4	7.4	7.5	7.5
480	6.0766	6.4752		5.3096	5.7612	5.7064	4.7723	5.7543
pH	7.0	6.6		7.5	7.6	7.7	7.8	
600			Contaminated	5.1732	5.5752	5.6335	4.0086	5.5922
pH				7.5	7.6	7.6	7.7	7.6
696	5.9445							
pH	6.7	6.6						
744				5.0531			2.7528	5.5159
pH					7.6		7.3	7.6
840	5.9777							
pH	7.0							
960				4.7033	4.6609	5.0569	0.0000	5.0864
pH				7.3	7.4	7.4	6.5	7.4
1200				4.5490	4.0294	4.1399	0.0000	4.9106
pH				7.5	7.6	7.5	7.0	7.5
1440				4.5092				
pH				7.7		7.7		

Note: The tap water was not subjected to high temperature to avoid dissolving glass constituents from containers.

Experiment 5. The effect of hydrogen ion concentration upon the mortality of Bact. typhosum for forty-eight hours in M/500 buffer solutions at 20°C.

TIME	pH 3.8		pH 5.0		pH 5.4		pH 6.4	
	I	II	I	II	I	II	I	II
hours								
0	5.3636	5.5682	5.7348	5.7825	5.7889	5.9036	5.8579	6.0000
3	2.3010	2.3010	5.6464	5.6212	5.7024	5.7686		
6	Sterile	Sterile	5.5623	5.5922	5.6875	5.6776	5.7118	5.7924
12			5.4425	5.4409	5.7059	5.7076	5.6637	5.7543
24			5.4031	5.4166	5.5038	5.7267	5.4548	5.5775
48			5.2041	5.0294	5.2672	5.3711	5.2253	5.3118
	VELOCITY COEFFICIENT, *k*							
	1.0209	1.0891	0.0110	0.0157	0.0109	0.0111	0.0132	0.0143

TIME	pH 7.1		pH 7.6		pH 8.7		pH 9.5	
	I	II	I	II	I	II	I	II
hours								
0	5.8195	5.6730	5.8482	5.8414	5.5922	5.7093	5.5752	5.5775
3					5.0088	5.1818	5.1139	4.8082
6	5.8899	5.6180	4.9699	4.6284	4.4829	4.2148	4.0128	3.8096
12	5.8555	5.5478	4.6628	4.5441	3.3010	2.4771	2.0000	2.3010
24	5.5809	4.3997	2.9420	2.6675	1.0792	0.0000	0.0000	0.3010
48	4.6435	2.6532	0.0000	1.1139	Sterile	Sterile	Sterile	Sterile
	VELOCITY COEFFICIENT, *k*							
	0.0244	0.0630	0.1215	0.0984	0.1887	0.2380	0.2979	0.2730

Experiment 6. Mortality of Bact. typhosum in M/500 buffer of pH 3.5 at different temperatures

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
minutes								
0	5.7760	5.8651	6.1004	6.0170	6.2856	5.9294	6.2041	5.9926
15					5.9106	5.4314	2.3617	2.0414
30			4.4533	4.1335	4.2695	4.6911	2.2625	1.6628
45					3.9854	3.6794	1.2788	1.0000
60	3.5065	3.8882	2.8865	3.3962	3.2455	3.1139	1.1461	0.6990
75						3.0000		
90			2.5911	2.9420				
120	1.8441	1.9294	1.9868	2.3032				
180	1.2788	1.0414	0.3010	0.3010				
240	1.1761	0.9777						
300	Sterile	0.0000						
	VELOCITY COEFFICIENT, *k*							
	1.1500	1.2219	1.9931	1.9053	3.0401	2.8155	5.0580	5.2936

Note: The buffer consisted of 50 cc. M/5 potassium acid phthalate plus 14.7 cc. M/5 HCl in 5000 cc. distilled water. This had a pH of 3.2 which shifted to 3.5 during sterilization.

Experiment 7. Mortality of Bact. coli in M/500 buffer of pH 8.5 at different temperatures

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
hours								
0	6.1847	6.2648	6.2304	6.2455	6.0344	6.2455	6.2227	6.1703
1							5.9309	6.0253
2					6.0212	6.0607	5.0128	5.6415
3							4.9699	5.2718
4	6.1553	6.2227	6.1703	6.0492	5.6675	5.9063	4.3636	4.7627
5							3.4456	3.9590
6							2.7186	2.9289
8	6.0828	6.2227	6.0899	5.9590	5.2175	5.5092	0.6021	
12	6.0334	6.1599	5.9699	5.7007	4.0864	4.2253		
15	6.0212	6.1761	5.9117	5.3139				
24	5.7846	5.9538	5.5092	4.9186				
32	5.5478	5.7796	5.2856	4.8055				
	VELOCITY COEFFICIENT, k							
	0.0199	0.0152	0.0295	0.0450	0.1623	0.1684	0.7026	0.5402

Note: The buffer solution was identical with that used in experiment 6.

Experiment 8. The mortality of Bact. coli at different temperatures in M/500 buffer at pH 6.1

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
hours								
0	6.2511	6.4578	6.0864	6.5824	6.4133	6.4249	6.1775	6.1790
24	6.1134	6.4305						
50	6.1271	6.4301						
74	6.0899	6.4085						
120	6.0846	6.4698	5.9180	6.5617	6.3858	6.3786	6.0676	6.1265
240	6.0933	6.4288	5.9325	6.5023	6.4171	6.3655	6.0660	6.0702
360	5.9357	6.2703	5.8848	6.4183	6.3416	6.2878	6.022	6.0212
480	5.9722	6.3049	5.7582	6.4232	6.3232	6.2844	5.9058	5.7042
600			5.8082	6.4198	6.2122	6.2148	5.5809	5.2625
720	5.8839	6.3219	5.7627	6.3670	6.2227	6.2068	5.3444	4.9047
840	5.9672	6.3959	5.6839	6.3579	6.0029	6.0128	5.2480	4.7679
960			5.7143	6.3619	6.1810	6.1422	4.9034	4.5587
1128			5.7152	6.4188	6.0745	6.0993	4.7185	4.3324
1230			5.6821	6.3275		6.0374	4.4440	4.1614
1440					5.9652			
1560			5.5416	6.1461	5.8998	5.8357	4.1072	3.8831
	VELOCITY COEFFICIENT, $k \cdot 10^2$							
			0.3454	0.2800	0.3292	0.3775	1.326	1.895

Note: The buffer consisted of 50 cc. M/5 KH_2PO_4 plus 4.75 cc. M/5 NaOH in 5000 cc. distilled water. The original pH of 5.9 shifted to 6.1 during sterilization.

Experiment 9. The mortality of Bact. coli at different temperatures in M/500 buffer at pH 7.1

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
hours								
0	6.3701	6.4752	6.3811	6.4514	6.5316	6.2405	6.3113	6.5051
25			6.3304	6.4440	6.4984	6.2896	6.2516	6.4786
48			6.3560	6.3574	6.4422	6.2350	6.2417	6.4735
74	6.3369	6.4734	6.2863	6.4019	6.4393	6.2054	6.2169	6.4824
120	6.2941	6.4914	6.3218	6.4084	6.4764	6.2222	6.2300	6.4433
240	6.3707	6.4436	6.2941	6.3516	6.4777	6.1945	6.1897	
384	6.3287	6.4541	6.3240	6.3104	6.3647	6.0682	6.0708	6.2601
480	6.3072	6.4254	6.2397	6.3118	6.3879	6.0792	5.8248	5.9643
600	6.3131	6.4079	6.3047	6.3843	6.2471	5.9294	5.4843	5.6712
840	6.2922	6.3728	6.1553	6.2989	6.2034	5.9015	5.0664	5.2363
984			6.1239	6.3560		5.8451	4.9395	5.2455
1200			6.1453		6.0998	5.5366	4.7723	5.0374
1440			6.1106	6.2227	5.9041	5.3874	4.5855	4.8785
	VELOCITY COEFFICIENT, k. 10^3							
	0.0928	0.1220	0.188	0.159	0.436	0.593	1.20	1.13

Note: The buffer consisted of 50 cc. M /5 KH_2PO_4 plus 29.63 cc. M /5 NaOH in 5000 cc. distilled water. The original pH of 7.0 shifted to 7.1 during sterilization.

Experiment 10. The mortality of Bact. coli at different temperatures in M/500 buffer at pH 8.0

TIME	0°C.		10°C.		20°C.		30°C.	
	I	II	I	II	I	II	I	II
hours								
0	6.1818	6.4526	6.3892	6.3485	6.5498	6.4439	6.4417	6.4882
24	6.1120	6.3141	6.2856	6.3464	6.3575	6.2923	6.2487	6.2777
48	6.1139	6.3395	6.2853	6.2889	6.3187	6.2350	6.1281	6.1351
75	6.0503	6.2413	6.2826	6.2084	6.2041	6.1383	5.9759	6.0374
120	6.0128	6.2227	6.2608	6.2209	6.1351		5.9309	6.0000
240	6.0212	6.2034	6.0958	6.1106	6.0212	5.9624	5.7016	6.0086
360	5.9542	6.2470	6.1367	6.1523	6.0425	5.7825	5.5250	5.8351
480	6.0029	6.2427	6.1470	6.1162	6.0107	5.7657	5.2504	5.6464
600			5.9881	6.0294	5.9661	5.6911	5.1875	5.4518
720	6.0238	6.2365	5.9445	6.0199	5.8865	5.5786	4.8825	5.3243
840			5.8651	5.9633	5.8451	5.5119	4.6503	5.2601
960			5.9206	6.0098	5.9143	5.6981	4.5453	5.2577
1200			5.9243	6.0029	5.8082	5.5933	4.0229	5.1430
1440			5.8938	5.9912	5.7745	5.3032	3.4787	3.7067
	VELOCITY COEFFICIENT, k. 10^3							
	0.2194	0.3141	0.3441	0.2841	0.5385	0.7925	2.058	1.933

Note: The buffer consisted of 50 cc. of M /5 KH_2PO_4 plus 46.80 cc. M /5 NaOH in 5000 cc. distilled water. After sterilization, the pH remained at 8.0. Toward the end of the experiment, the pH gradually shifted to 7.7, apparently because of the low concentration of the buffer and the nearness to the limit of its zone of effectiveness.

Experiment 11. Mortality of Bact. coli in M/500 buffers at 20°C.

TIME	pH 8.4		pH 8.9		pH 9.5	
	I	II	I	II	I	II
hours						
0	6.5391	6.2584	6.3874	6.4564	6.1818	6.2577
24	6.5159	6.2430	6.3973	6.3079	6.1703	6.1206
48	6.4683	6.2414	6.3784	6.4502	6.0719	6.0294
72	6.6021	6.2492	6.4031		6.0374	6.0607
120	6.4969	6.1614	6.4216	6.4654	6.0607	5.9638
264	6.4393	6.2072	6.4089	6.4314	5.9890	6.0086
480	6.5809	6.2227	6.5172		5.9890	6.0098
624	6.5539	6.2227	6.5441	6.4624	5.9912	5.7973
840	6.4955	6.2201	6.4133	6.3711	5.8494	5.8573
	pH shifted to 7.9					

Experiment 12. The mortality of Bact. typhosum in autoclaved tap water at 20°C.

TIME		I	II	III
hours				
0		6.5873	6.5628	6.7284
	pH	8.9	8.7	8.4
6		6.5463	6.5721	6.6887
	pH	8.9	8.7	8.4
24		6.4742	6.5575	6.6939
	pH	9.0	8.8	8.8
48		5.1931	6.5968	6.6532
	pH	8.9	8.8	8.7
72		3.4914	5.2742	6.6135
	pH	8.9	8.7	8.7
96		2.6096	4.9074	6.5064
	pH	8.9	8.7	8.6
120		2.4065	4.6375	6.4389
	pH	8.9	8.7	8.5
144		2.2201	4.7771	6.2695
	pH	8.9	8.7	8.6
167		1.9243	4.2380	6.1804
	pH	8.9	8.5	8.5
216		1.1139	3.6021	5.6821
	pH	8.5	8.3	8.2
264		0.6990	5.7275	5.5159
	pH	8.2	7.7	7.6
312		Sterile	5.6096	5.2684
	pH	8.1	7.9	7.9
384			5.4654	4.9299
		8.3	8.1	8.1

Experiment 13. Mortality of Bact. typhosum in M/500 buffer of pH 6.3–6.5 at 20°C.

TIME	PHOSPHATE BUFFER			PHTHALATE BUFFER		
	I	II	III	I	II	III
hours						
0	6.6133	6.5376	6.5417	6.6073	6.5234	6.4469
6	6.6314	6.5703	6.5931	6.6703	6.5289	6.4510
24	6.6583	6.5090	6.5916	6.6086	6.5489	6.4713
48	6.5991	6.5714	6.5895	6.6074	6.4821	6.4004
72	6.5705	6.5907	6.5844	6.6425	6.4938	6.4327
96	6.6087	6.5609	6.5585	6.5821	6.5314	6.4284
120	6.5627	6.5038	6.4813	6.4846	6.4705	6.4480
144	6.5714	6.5502	6.5370	6.5555	6.4548	6.3720
167	6.5991	6.5658	6.5658	6.4705	6.4702	6.3988
216	6.5363	6.5502	6.3375	6.4975	6.3756	6.3290
264	6.5647	6.5563	6.5922	6.5350	6.4487	6.4294
312	6.6206	6.5723	6.6157	6.5488	6.4932	6.3639
384	6.5899	6.5447	6.5465	6.5798	6.5452	6.4396
552	6.2876	6.4976	6.6204	6.4343	6.3897	6.2819
763	5.5872	5.9064	5.9694	5.9890	6.0174	5.6945
960	5.0515	5.3345	5.7179	5.7669	5.8873	5.2978

Note: The Clark and Lubs phosphate and phthalate buffers of pH 6.0 were diluted to M/500 and autoclaved, after which their pH remained between 6.3 and 6.5. This experiment was performed six months after Nos. 1–11 and shows that any specific effect of the buffer as a salt is subordinate to the pH effect, at least under these conditions.

MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

I. INTRODUCTORY[1]

SELMAN A. WAKSMAN

Received for publication June 16, 1921

Hydrogen sulfide and other sulfides in solution are slowly oxidized to sulfur, under natural conditions. The sulfur formed, as well as elementary sulfur, particularly when present in a fine state of subdivision and in the presence of certain catalytic agents, undergoes further oxidation, the resulting product being sulfuric acid. In the presence of specific bacteria, this phenomenon is much more rapid. These bacteria were first studied extensively by Winogradsky and were designated by him as sulfur bacteria; they possess a strong oxidizing power, in contradistinction to the reducing bacteria, which form hydrogen-sulfide from sulfur and its compounds. The sulfur bacteria, originally studied by Winogradsky, contain sulfur granules within their cells, as a result of the oxidation of the hydrogen sulfide. Later investigators, in their studies of sulfur oxidation, also included, under the term sulfur bacteria, organisms which are able to oxidize hydrogen sulfide, sulfur, and thiosulfate, but which do not, however, store any sulfur within their cells.

The so-called sulfur bacteria are not related to one another morphologically and belong to widely different genera. By the use of physiological and morphological differences, they can be divided into five groups: (1) colorless, thread forming bacteria, accumulating sulfur within their cells. (2) Colorless, non-thread-forming bacteria, accumulating sulfur within their cells. (3) Purple bacteria, oxidizing sulfur and accumulating it within

[1]Paper No. 32 of the Technical Series, New Jersey Agricultural Experiment Station, Department of Soil Chemistry and Bacteriology.

their cells. (4) Bacteria oxidizing sulfur and sulfur compounds, but accumulating sulfur outside their cells. (5) Bacteria oxidizing elementary sulfur and not accumulating any sulfur within or without their cells. The first four groups are mentioned in the latest texts and reviews on sulfur bacteria, while the fifth group has been suggested on the basis of the results presented below.

It is of interest to note that in most of the work on sulfur oxidation by bacteria, with the exception of that of Jacobsen (1912),[2] the starting point was not sulfur itself, but hydrogen sulfide, sulfides, or thiosulfates. The activity of the organisms was judged not by the oxidation of sulfur, as measured by the production of sulfuric acid and sulfates and subsequent change in reaction, but either by the disappearance of the sulfide in the medium or by the appearance or disappearance of the sulfur granules within or without the microbial cell. Of importance is also the fact that all the earlier work and most of the later work has been done with organisms present in canal water, mud water, curative muds, and sea water and very little attention was paid to the microörganisms concerned in the oxidation of sulfur in the soil. This is the reason for using hydrogen sulfide and sulfides as a source of sulfur, since the latter substance, and not elementary sulfur, is present or is produced abundantly under those conditions.

A detailed review of the oxidation of sulfur and sulfur compounds by microörganisms is given in the papers of Omeliansky (1904), Düggeli (1919) and in the book by Kruse (1910). A brief historical review is presented here for a better understanding of the work that is to follow.

The first group of sulfur oxidizing bacteria (colorless, thread forming) consists of three genera: Beggiatoa, motile, forming no sheaths; Thiothrix, fastened, forming no sheaths, and Thioploca, threadforming bacteria, surrounded with a jelly-like sheath. The Beggiatoas were the first organisms to attract attention as having to do with the oxidation of sulfur or its derivatives. Cramer (1870) pointed out that the granules found

[2] Bibliography is found at the end of the second article, in this series.

within the cells of Beggiatoa consisted of sulfur. Cohn (1875) then proposed the theory that the Beggiatoa and the purple bacteria produce hydrogen sulfide by the reduction of sulfates. But it was Winogradsky (1883, 1887, 1888) who demonstrated that the hydrogen sulfide produced by other bacteria is oxidized by the Beggiotoa to sulfur and sulfuric acid.

$$2H_2S + O_2 = 2H_2O + S_2 + 122 \text{ Cal.}$$
$$S_2 + 3O_2 + 2H_2O = 2H_2SO_4 + 282 \text{ Cal.}$$

This oxidation is so important for the very existence of these organisms that, when the hydrogen sulfide is taken out of the medium, they oxidize the sulfur present within their cells and, when this is used up, they die out. The presence of traces of organic substances and nitrates in the water is sufficient for the development of these organisms, as long as there is enough hydrogen sulfide, while the presence of sugars, peptone and like nutrients will stimulate the growth of other microbes but will injure these sulfur bacteria.

According to Winogradsky (1888), the sulfuric acid formed is neutralized by the calcium carbonate present in the water.

$$H_2SO_4 + CaCO_3 = CaSO_4 + CO_2 + H_2O$$
$$H_2SO_4 + CaH_2(CO_3)_2 = CaSO_4 + 2CO_2 + 2H_2O$$

The reaction of the water cultures of the acid producing bacteria was not found to become acid. Beggiatoa has been obtained in pure culture by Keil (1912). No definite physiological studies were made by Winogradsky. In general, the results of this investigator are summarized under the following four headings: (1) The sulfur bacteria oxidize hydrogen sulfide and accumulate sulfur in the form of small spheres, consisting of soft amorphous sulfur which never crystallizes in the living cells. (2) They oxidize the sulfur to sulfuric acid, which is at once neutralized, by the carbonates present, into sulfates. (3) Without sulfur, the organisms soon die off. (4) They can live and multiply in liquids containing only traces of organic substances.

This last point was refuted by Keil (1912), who demonstrated that the organisms are autotrophic and do not need organic

substances for their growth. Keil claims to have isolated pure cultures of Beggiatoa and Thiothrix, and found these organisms to be able to live in media free from any traces of organic matter, although the presence of small quantities of organic substances is not detrimental to these organisms. Ammonium salts are used as sources of nitrogen and only carbonic acid as a source of carbon. Carbon dioxide pressure may vary within the limits of 0.5 and 350 mm. (25 mm. is the optimum); oxygen may vary within 10 to 20 mm. and H_2S within 0.6 to 1.7 mm. The presence of carbonates is important for the neutralization of the acids. The organism seems to assimilate carbon at the rate of 1 gram per 8 to 10 grams of sulfur oxidized (see also Hinze and Molisch). The Thioplaca has been studied in detail by Wisloch and Kolkwitz.

The second group of the sulfur oxidizing bacteria consists of colorless organisms forming no threads and containing sulfur within their cells. The following forms belong to this group: *Monas* (Hinze (1913)), *Thiophysa* (Hinze (1903)), *Thiovulum* (Hinze (1913)), *Spirillum* (Molisch (1912)), *Thiospirillum* (Omelianski (1905)), *Bacterium bovista* (Molisch (1912)), *Bacillus thiogenes* (Molisch (1912)), and *Achromatium* (Nadson (1903), and Griffith (1913)).

Some idea of this group of organisms is obtained from a reference to the work of a few investigators. Jegunow (1896) studied the oxidation of the hydrogen sulfide formed in the mud of the Liman in Odessa and in the Black Sea. He described two sulfur bacteria; Thiobacterium α, a motile, colorless, slightly curved organism, 4.5 to 9μ long and 1.4 to 2.3μ wide, containing a finely granulated plasma and large sulfur granules. Thiobacterium β, motile, colorless, curved, 2.5 to 5 by 0.6 to 0.8μ, and containing a row of shining sulfur granules. *Monas Mülleri* was described in detail by Hinze (1913), who has shown that this organism belongs morphologically to the flagellates and physiologically to the sulfur bacteria. Most of these organisms were isolated from the water.

Gicklehorn (1920) recently described several new sulfur bacteria, of which two are classified with this group, namely: *Spiril-*

lum agilissimum isolated from river mud in Gratz which measures about 6 to 10 by 1.8 to 2μ, of a rapid motility,and filled with black sulfur granules; *Chromatium cuculliferum* which is round to slightly elliptical, 6 by 4μ, of a slow motility, with black, shining, sulfur drops always found in one pole, with one flagellum on the granule-free pole. This form was found in a rotting mass of algae in the garden basin at Gratz. Gicklehorn also described three more forms: *Microspira vacillans*, *Pseudomonas bipunctata* and *Pseudomonas hyalina*, observed in the slime of the large basin in the botanical garden at Gratz, which he classified with the colorless sulfur bacteria. It must be noted here that none of the forms studied by Gicklehorn were cultivated in pure culture, which is true of most of those studied by the other investigators.

The third group of the sulfur oxidizing organisms is found among the purple bacteria. These are distinguished from the sulfur bacteria described above by the production of a red, red violet or red brown pigment which is unevenly distributed throughout the cell. In addition to the red pigment (bacterio-purpurin), there is also present in all these bacteria a green pigment (bacterio-chlorin). These bacteria are found abundantly in sulfur springs and in mud waters. Not all the purple bacteria are able to utilize hydrogen sulfide. Molisch (1907) succeeded in cultivating some of them in pure culture, but not the sulfur forms. The rôle of sulfur in the metabolism of purple bacteria is still an open question, since, according to Nadson (1903) and Molisch (1907), the hydrogen sulfide is not required for nutrition and sulfur is not accumulated. These results are in direct opposition to the earlier ideas of Winogradsky (1888) and others. A detailed study of the purple bacteria is found in the work of Molisch (1907).

The fourth group of sulfur bacteria includes colorless organisms that do not accumulate sulfur within their cells. These were first demonstrated by Nathanson (1903) in sea water, and were found to be able, by means of oxidation of hydrogen sulfide or sodium thiosulfate, to reduce carbonic acid and construct from it organic substances. By using a medium consisting of 3 per cent NaCl, 0.25 per cent $MgCl_2$, 0.1 per cent KNO_3, 0.05 per cent

Na_2HPO_4, 0.2 to 1 per cent $Na_2S_2O_3$ and some $MgCO_3$, he obtained a good growth of these bacteria and, on adding agar, he has been able to isolate them in pure culture. In the absence of the carbonate, but in the presence of CO_2 containing air, the growth was much slower; in the absence of both carbonate and CO_2, no growth took place, even in the presence of various organic substances. The medium did not become acid even in the absence of carbonate, indicating that the oxidation of the thiosulfate did not take place according to the formula:

$$Na_2S_2O_3 + H_2O + 4O = Na_2SO_4 + H_2SO_4.$$

but according to the following reaction:

$$3Na_2S_2O_3 + 5O = 2Na_2SO_4 + Na_2S_4O_6.$$

While no sulfur accumulates within the cell, there is an abundant production of free sulfur outside of the cell, not in direct contact with the colony, but at some distance from it, suggesting an extracellular oxidation.

Beijerinck (1904) confirmed the results of Nathanson (1903) by the use of a medium consisting of 100 parts of water, 0.5 $Na_2S_2O_3$. 5 H_2O, 0.1$NaHCO_3$, 0.02 K_2HPO_4, 0.01 NH_4Cl and 0.01 part $MgCl_2$. The medium was not sterilized, was inoculated with canal water and incubated at 28 to 30°. In 2 to 3 days, the surface of the medium became covered with free sulfur, filled with bacteria. On making a transfer into a fresh flask with medium, a sulfur layer was obtained in 24 hours. According to Beijerinck (1904), the reaction takes place as follows:

$$Na_2S_2O_3 + O = Na_2SO_4 + S.$$

This reaction is exothermic and functions as a source of energy, which is used for the reduction of $NaHCO_3$ and for the building of the bacterial body. CaS and H_2S can replace the thiosulfate. $H_2S + O = H_2O + S$.

$$Na_2S_4O_6 + Na_2CO_3 + O = 2\,Na_2SO_4 + CO_2 + S_2$$

The ammonium salt can be replaced by nitrates. No other of the organic substances tested could replace the carbonic acid as

a source of carbon. The organism, *Thiobacillus thioparus*, is a short rod, 3 by 0.5μ, not forming any spores, very motile and very sensitive, so that on plates the organisms die off in a week.

By using a medium consisting of canal water 100, powdered sulfur 20, KNO_3 – 0.5, Na_2CO_3 – 0.02, $CaCO_3$ – 2.0, K_2HPO_4 – 0.02 parts, in a closed flask, incubated at 30°C., Beijerinck obtained an oxidation of sulfur accompanied by a reduction of the nitrate to atmospheric nitrogen.

$$6KNO_3 + 5S + 2\,CaCO_3 = 2K_2SO_4 + 2\,CaSO_4 + 2\,CO_2 + 3N_2 + 695.5\ \text{Cal.}$$

The sulfur is oxidized to sulfuric acid which acts upon the $CaCO_3$ giving $CaSO_4$ and CO_2. By using a medium consisting of tap water – 100, $Na_2S_2O_3$. 5 H_2O – 0.5, K_2HPO_4 – 0.01, $NaHCO_3$ – 0.02, agar 2.0 parts, Beijerinck isolated, in pure culture, the organism, *Thiobacillus denitrificans*, which is a very motile, short rod, hardly distinguishable microscopically from *Thiobacillus thioparus*. *Thiobacillus denitrificans* was further studied by Lieske (1912) and Gehring (1915) and was found to occur in various soils. The organisms on the plate, lose their ability to grow rapidly, long before they are dead. Beijerinck's work was continued further by Jacobsen (1912, 1914), who found a crude culture of *Thiobacillus thioparus* to be able to oxidize 58.8 mgm. of sulfur to sulfuric acid, in five weeks, out of a total of 648 mgm. added to the medium. Pure cultures oxidized, in eight weeks, 165 mgm. out of 648 mgm. of sulfur added.

Gicklehorn (1920) has studied two organisms belonging to the fourth group of sulfur-oxidizing bacteria, found in garden soil, which are able to oxidize K_2S with the liberation of free sulfur. The organisms are 1 to 2 by 0.3 to 0.5μ and 2 to 4 by 0.5 to 1μ in size. However, he did not isolate his organisms in pure culture and did not record any quantitative physiological data.

Finally, we have a fifth group of sulfur bacteria, which are studied in detail in the next paper. Two preliminary reports on this organism by Waksman and Joffe (1921a, 1921b) and a detailed study of the methods used in its isolation, by Lipman, Waksman and Joffe (1921), were published elsewhere.

To summarize: (1) The microörganisms concerned in the sulfur cycle are separated into reducing bacteria and oxidizing bacteria, the latter being the true sulfur bacteria. (2) The true sulfur bacteria are divided into five groups: the first three groups of sulfur bacteria are found in sulfur springs, canal and mud waters, curative muds, river water and sea water; they oxidize hydrogen sulfide and sulfides, but not elementary sulfur, and accumulate sulfur within their cells; the fourth group of bacteria, consisting of small rod shaped organisms, is found in sea water, canal water and soil; these bacteria are able to oxidize hydrogen sulfide and other sulfides, thiosulfates and elementary sulfur, forming a heavy pellicle on the surface of the medium and allowing an accumulation of sulfur outside of their cells; the fifth group of sulfur bacteria occurs in soils to which elementary sulfur has been added, particularly in soil-sulfur-composts, oxidizing primarily elementary sulfur, thiosulfates to a small extent, but not hydrogen sulfide or sulfides; these bacteria grow uniformly throughout the medium, not forming any pellicle, do not liberate any sulfur and allow a very intensive production of sulfuric acid, and the necessary carbon is derived entirely from the carbon dioxide of the atmosphere; the fifth group is morphologically related to group four, but includes organisms very small in size and the strongest sulfur oxidizing and acid producing bacteria known.

MICROÖRGANISMS CONCERNED IN THE OXIDATION OF SULFUR IN THE SOIL

II. THIOBACILLUS THIOOXIDANS, A NEW SULFUR-OXIDIZING ORGANISM ISOLATED FROM THE SOIL[1]

SELMAN A. WAKSMAN AND J. S. JOFFE

Received for publication June 16, 1921

By composting sulfur, rock phosphate and soil it was found (McLean, 1918) that sulfur is rapidly oxidized to sulfuric acid; the acid acts upon the tricalcium phosphate, converting it into di- and mono-calcium salts. In the absence of a neutralizing agent or, after this agent has all been used up, the sulfuric acid formed, in the presence of an excess of sulfur, accumulates in the medium. On inoculating such composts into proper culture media, we finally succeeded in isolating a small bacterium which is active in the oxidation of the sulfur. A detailed study of the composting of sulfur, of the transformation of the tri-calcium phosphate and of the methods used in the isolation of the organism are found elsewhere (Lipman, Waksman and Joffe, 1921); only a brief review of the process of isolation is presented here.

Method of isolation. The following media were originally used for the isolation of the organism:

Medium 1:

$(NH_4)_2SO_4$	2.00 gram
K_2HPO_4	1.00 gram
$MgSO_4$	0.50 gram
KCl	0.50 gram
$FeSO_4$	0.01 gram
Sulfur	10.00 grams
$Ca_3(PO_4)_2$	10.00 grams
Distilled water	1000.00 cc.

Medium 2: Same as no. 1, but with 0.1 per cent glucose.

Medium 3: Same as no. 1, but in place of 10 grams only 2.5 grams $Ca_3(PO_4)_2$ per liter.

---

[1]Paper No. 33 of the Technical series, New Jersey Agricultural Experiment Stations, Department of Soil Chemistry and Bacteriology.

The media were distributed in 100 cc. portions into 250 cc. Erlenmeyer flasks and sterilized in flowing steam, for 30 minutes, on three consecutive days. The flasks were then inoculated with various dilutions of the composts. Medium 2 was found to allow a growth of both a sulfur oxidizing bacterium and one or more species of fungi. By omitting the glucose from the medium, the fungi were practically eliminated.

It was found later that, by cutting down the tri-calcium phosphate in the medium to 0.25 per cent, a more rapid development of the organism took place, thus giving medium 3, which is a modification of 1 and 2.

Well advanced composts were used for inoculation. The material was diluted 10, 1000, 100,000 and 10,000,000 times with sterile water, then 1 cc. of each dilution was added to 100 cc. of the sterile medium and the flasks incubated, at 25°, for seven to fourteen days.

The flasks became turbid on the fourth or fifth day, the amount of turbidity depending upon the dilutions used, the higher dilutions developing slower than the lower ones. A pellicle or fungus mycelium was formed only in the flasks containing glucose. By transferring the cultures into fresh flasks, the same phenomenon was observed with a uniform turbidity in four to five days. By examining the culture under the microscope, it was found to contain a very minute non-motile bacterium present in abundance and accompanied by a few larger cylindrical cells which were found to be spores of a fungus occurring abundantly in the compost. The impure culture of the organisms was found to possess strong sulfur-oxidizing properties, about 200 to 300 mgm. of the sulfur being oxidized, in each flask, in fourteen days. In the presence of tri-calcium phosphate more of the sulfur is oxidized, since the acid formed is used up in converting the insoluble phosphate into soluble calcium-acid-phosphate and calcium sulfate. A further accumulation of the sulfuric acid resulted also in the formation of phosphoric acid and calcium sulfate. The medium had originally a reaction equivalent to pH 5.6 to 6.2. Following the oxidation of the sulfur, the reaction became gradually acid and, at a pH of

2.6–2.8, the reaction remained stationary till all the tri-calcium phosphate had been transformed into mono-calcium salt, after which the reaction became more acid, as shown in table 1 and figure 1.

All attempts to grow the sulfur-oxidizing organisms on solid media failed, neither agar nor silica-jelly media allowing any growth to take place.

TABLE 1

Course of reaction and accumulation of water soluble phosphates

AGE OF CULTURE	pH	PER CENT OF INSOLUBLE PHOSPHATES MADE WATER SOLUBLE*
days		
At start	5.4	
1	5.4	
2	5.3	0.9
4	4.6	5.5
6	3.5	
8	2.6	33.7
10	2.7	27.5
12	2.6	81.7
15	2.4	93.9
19	2.3	86.3
23	2.3	85.9
30	1.8	
38	1.8	86.1
68	1.7	
120	0.8	85.9

*Medium contains originally 1 per cent insoluble phosphate.

A pure culture was obtained by continued transfer in fresh flasks with high dilutions, so as to eliminate any contaminating organism, the medium being made acid at the start (pH 2.0–3.0), by the use of phosphoric acid and mono-potassium phosphate. The culture was finally obtained in a pure state. Its purity was demonstrated by repeated microscopic examinations, by the uniform growth in the liquid media and by the fact that no organism developed, when the culture was inoculated upon common bacteriological media.

On repeated transfer, the culture was found to deteriorate since it took a longer period of time to develop. It was found, necessary, in order to obtain a good growth, to use a sterile pipette instead of a loop, one or two drops being sufficient to inoculate 100 cc. By buffering the medium with suitable substances, such as phosphates, the organism would develop much more rapidly, particularly at the more acid reactions. The

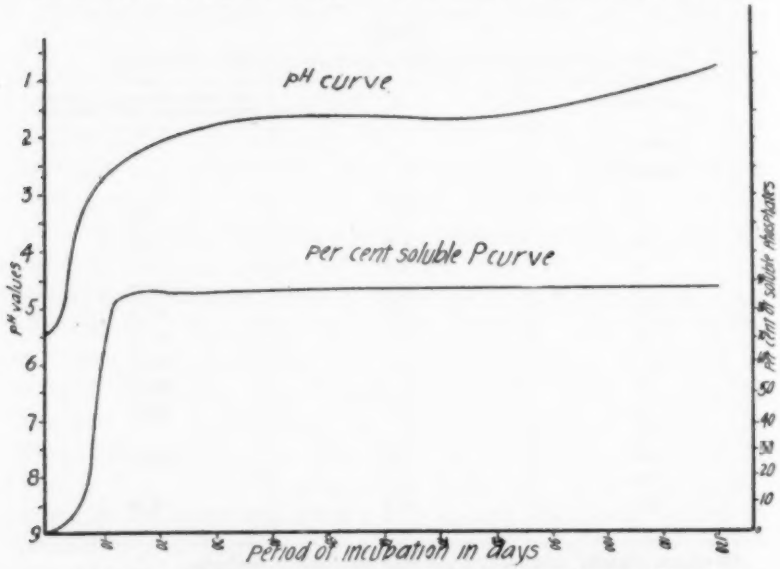


FIG. 1. COURSE OF REACTION AND ACCUMULATION OF WATER SOLUBLE PHOSPHATES IN A PURE CULTURE OF *Thiobacillus thiooxidians* N. SP.

organism was found to be morphologically similar to the two Thiobacilli described by Beijerinck, and it is, therefore, classified in that genus, under the name of *Thiobacillus thiooxidans* n. sp.

Morphology. Vegetative cells, on the synthetic media used, are short rods, with rounded ends, usually occurring singly, to some extent in pairs and rarely in triplets. The majority are less than 1 micron long and about 0.5 micron in diameter. Spore formation, absent. The majority of the cells are non-motile, although a few motile cells can also be found in young (seven

days old) cultures. The organism stains well with gentian-violet and methylene blue. It is Gram-positive.

CULTURAL CHARACTERISTICS

No agar or other solid medium has been found as yet, upon which the organism would grow. It grows in liquid media with a strong uniform clouding, without any surface growth or sediment formation. It does not grow on the common organic media, although the presence of glucose or peptone in the medium is not injurious. Inorganic media containing sulfur as a source of energy are suited for its growth. In the presence of tri-calcium phosphate, the growth of the organism is accompanied by characteristic reactions: the sulfur forming originally a layer on the surface of the medium usually drops to the bottom, the sulfuric acid formed from the oxidation of the sulfur dissolves the tri-calcium phosphate giving soluble phosphate and $CaSO_4 \cdot 2H_2O$, the calcium sulfate crystallizes out in the form of radiating monoclinic crystals hanging down from the sulfur particles that are floating on the surface of the medium or protruding upward from the bottom. The reaction of the medium becomes acid as indicated by the change in the hydrogen-ion concentration. At a pH of about 2.8, the reaction becomes stationary till all the calcium-phosphate has been dissolved. In the presence of an excess of this neutralizing agent, or in the presence of rapidly dissolving alkaline carbonates, the culture is injuriously affected. Anything that will tend to change the medium to an alkaline or even a less acid reaction (except, of course, the action of the buffers), such as shaking the culture, in the presence of even smaller amounts of tri-calcium phosphate, will also tend to affect the uniform growth of the organism injuriously.

The culture can be kept alive for numerous consecutive generations on the liquid media and when not injured by an excess of alkali or acid, may be as active as a recently isolated culture.

The index No. of *Thiobacillus thiooxidans* is, according to the new Descriptive Chart of the Society of American Bacteriologists, 5332–5230–2222.

PHYSIOLOGY

Source of carbon. The organism derives all its carbon need from the CO_2 of the atmosphere. When carbon was introduced into the culture in the form of carbonates and bicarbonates, the presence of the former prevented growth due to the fact that they kept the medium alkaline, thus preventing a normal development of the organism, while the latter, if present only in small amounts, allowed a good growth to take place. But since the growth was not any better, and to some extent even worse than in the bicarbonate-free flasks, its use is superfluous. At this point, we get a clear differentiation in the metabolism of two important autotrophic organisms, the nitrifying and the sulfur-oxidizing bacteria. While the former thrives best at an alkaline reaction, the latter grow best at an acid reaction. Sodium bicarbonate is considered to be indispensable for the nitrifying bacteria; this was thought to be due to the utilization of the bicarbonate as a source of carbon, but, as recently pointed out by Meyerhof (1916), the bicarbonate merely serves the purpose of a buffer in the medium, to keep the reaction alkaline (optimum pH 8.3–9.3). In the case of the sulfur oxidizing bacterium, which has its optimum at a distinctly acid reaction (pH 3.0–4.0), the bicarbonate is not necessary since its buffering properties will tend to make the medium less acid and thus have an injurious effect, while as a source of carbon, the CO_2 from the atmosphere seems to be sufficient.

Source of energy. Sulfur is the all important source of energy for this organism. The organism is strictly autotrophic and, although glucose did not exert any injurious action, and perhaps its action was even to some extent beneficial, the amount of sulfur oxidized and acid produced were about the same in glucose and in glucose-free cultures.

In addition to sulfur, thiosulfate is also utilized, but to a much smaller extent: while, with elementary sulfur, growth appears in four to five days, under favorable conditions, as demonstrated by the turbidity and change in pH value, with thiosulfate, growth appears only in ten to twelve days and is much slower.

Hydrogen sulfide and sulfides are not utilized at all, which sharply differentiates our organism from those of Nathanson (1903), Beijerinck (1904), and Jacobsen (1914), as will be pointed out later.

Mineral requirements. Mere traces of K, Mg, Ca, Fe, in addition to phosphates, are sufficient for the growth of the organism. As a matter of fact, good growth and good sulfur oxidation were obtained by omitting, in various batches of media, each of the first four minerals, but, of course, no precaution was taken to eliminate any traces present in the distilled water or any substances that might have been dissolved out by the action of the sulfuric acid on the glass of the flask.

Source of nitrogen. Due to the very small amount of growth made by the organism, the amount of nitrogen required is very small: without introducing any nitrogen source into the medium, some growth is obtained, the nitrogen being derived either from the contamination of the other salts, the distilled water, or traces of ammonia in the atmosphere. The best sources of nitrogen are ammonium salts of inorganic acids (particularly sulfate), followed by the ammonium salts of organic acids, after which come the nitrates, asparagin and amino acids. Nitrites, in concentrations used (2 grams per liter) are toxic. Good growth is obtained with pepton, but the amount of sulfur oxidized is less than with the other sources of nitrogen.

Relation to oxygen. The organism is strictly aerobic, in view of the fact that it derives the oxygen necessary for the oxidation of sulfur to sulfuric acid from the atmosphere.

Influence of organic substances. As pointed out above, glucose does not act injuriously, neither do other organic substances, like pepton. Substances like glycerol, alcohol, mannitol and glucose seem to have a slight favorable effect in the presence of a good nitrogen source. All these substances either act like stimulants or else take part in the structural requirements of the organism.

Influence of stimulants. In addition to the pure organic substances, above mentioned, which may stimulate to some extent the growth of the organism, other substances may exert

the same action. A detailed study of the influence of stimulants on the oxidation of sulfur by a pure culture of *Thiobacilllus thiooxidans* is presented in table 2.

The medium was buffered with phosphoric acid and monopotassium-phosphate to a pH of about 3.0 It was distributed

TABLE 2

Influence of stimulants on the oxidation of sulfur

$Ca_3(PO_4)_2$ 0.25 PER CENT	STIMULANT, 0.1 PER CENT	TOTAL SULFATES IN 100 cc. OF MEDIUM MILLIGRAMS	pH	TITRATION*
		mgms.		
+	Control	241.0	3.0	1.0
+		1476.0	1.4	2.8
−		912.5	1.5	2.5
	$CaSO_4$ (0.25 per cent)	1041.0	1.4	2.7
+	Glucose	991.0	1.5	2.7
−	Glucose	1012.5	1.3	2.6
+	Mannitol	1008.0	1.6	2.5
−	Mannitol	859.0	1.5	2.5
+	Glycerol	858.2	1.6	2.5
−	Glycerol	948.5	1.4	2.6
+	Alcohol	905.0	1.6	2.5
−	Alcohol	994.0	1.4	2.8
+	Soil	1019.5	1.6	2.7
−	Soil	1226.8	1.4	2.6
+	$Al_2(SO_4)_3$	1394.0	1.4	2.9
−	$Al_2(SO_4)_3$	989.0	1.4	2.6
+	Thallium nitrate	1133.1	1.5	2.7
−	Thallium nitrate	883.5	1.5	2.5
+	$MnSO_4$	1013.4	1.6	2.5
−	$MnSO_4$	933.8	1.4	2.5

*Titration = cubic centimeter of $\frac{N}{10}$ NaOH necessary to neutralize 1 cc. of culture, with phenolphthalein as an indicator.

in 100 cc. portions in 250 cc. Erlenmeyer flasks containing 1 gram portions of powdered sulfur and the proper amounts of $Ca_3(PO_4)_2$, where present. The flasks were plugged with cotton and sterilized in flowing steam, for thirty minutes, on three consecutive days. The organic substances were sterilized separately, then added to the sterile medium. The flasks were all inoculated with one drop of the same pure culture and incubated

for twelve days. At the end of that period, the pH was determined by the colorimetric method, titration was obtained from the amount, in cubic centimeters, of $\frac{N}{10}$ NaOH necessary to neutralize 1 cc. of the filtered culture using phenolphthalein as an indicator. The total sulfates were obtained by adding the amounts of soluble and insoluble sulfates: the latter were obtained by digesting the filtered residue in acidulated water and determining the sulfates in an aliquot portion.

In the presence of calcium phosphate, the largest amount of sulfur oxidized by a pure culture of the organism was obtained in medium 1, to which no stimulating agent has been added. In the absence of the tri-calcium phosphate, the amount of sulfur oxidized was appreciably less, oxidation in this case being stimulated by various substances. The most beneficial influence was exerted by the addition of a small amount of soil: this may be due to the introduction, with the soil, of a small amount of the lacking calcium salt or of some vitamine-like substance. The favorable action of the organic substances, aluminum and manganese sulfates, may be of a stimulating nature; however, this beneficial action is only very small and lies within the range of natural variability of the organism.

Influence of temperature. The optimum temperature for the activities of *Thiobacillus thiooxidans* n. sp. lies at about 28° to 30°C. Growth and sulfur oxidation are much slower at lower temperatures (18°) and at 37°C. Temperatures of 55°–60°C. are sufficient to kill the organism.

THE NATURE OF ACID FORMED AND THE INFLUENCE OF REACTION UPON THE GROWTH OF THIOBACILLUS THIOOXIDANS N. SP.

To get an insight into the true nature of the acid formed, particularly in the presence of tri-calcium phosphate, a series of tubes containing 2 cc. portions of the culture were arranged; measured quantities of $\frac{N}{1}$ NaOH were added to these, then the volume of the liquid was brought, in all tubes, to 3 cc. by the addition of distilled water. The hydrogen ion concentration of the tubes was then determined, by the colorimetric method. The results are tabulated in table 3 and graphically presented in figure 2.

TABLE 3

Titration and hydrogen-ion concentration of a 14 day old culture of Thiobacillus thiooxidans n. sp.

$\frac{N}{1}$ NaOH	pH VALUES	
	No calcium phosphate in the original culture	0.25 per cent of tri-calcium phosphate originally present in the culture
cc.		
0	1.5	1.5
0.02	1.6	1.5
0.04	1.7	1.6
0.06	1.7	1.7
0.08	1.7	1.7
0.10	1.8	1.7
0.12	1.8	1.8
0.14	1.9	1.8
0.16	2.0	1.9
0.18	2.0	1.95
0.20	2.2	2.0
0.22	2.3	2.2
0.24	2.4	2.2
0.26	2.5	2.3
0.28	2.6	2.4
0.30	2.8	3.0
0.32	4.4	3.6
0.34	6.4	4.4
0.36	6.4	5.6
0.38	6.4	6.2
0.40	6.4	6.6
0.42	6.4	6.6
0.44	6.6	6.6
0.46	6.6	6.6
0.48	7.4	6.6
0.50	7.5	6.6
0.52	9.0	7.2
0.54	9.4	7.2
0.56		8.8
0.58		9.6

It will be observed, by glancing at the curves in figure 2, that the hydrogen-ion concentration slowly decreases, as manifested by a slow increase in the pH values, with the addition of alkali, till the pH reaches 2.8, then there is a sudden drop in the curve, to pH 6.4, when the curve again becomes slanting, followed by a second drop. This gives the buffer effect of the cultures; the buffer action is more pronounced in the presence of tri-calcium phosphate, which increases the phosphate content of the medium.

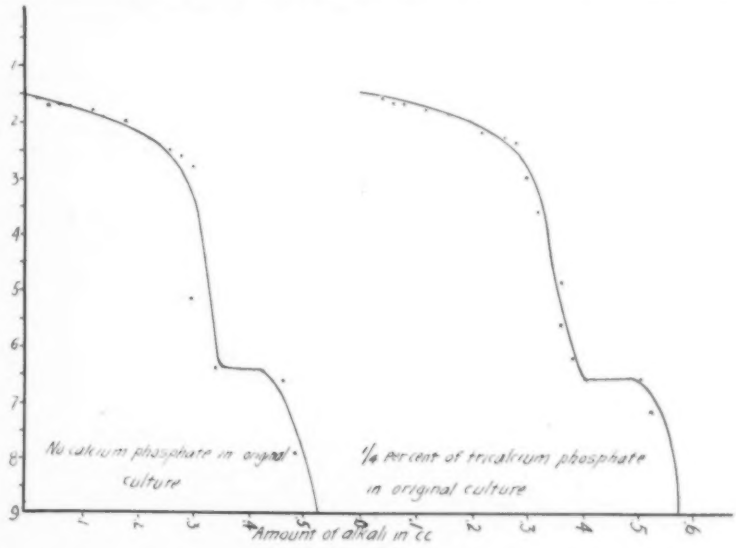

No calcium phosphate in original culture
1/4 Per cent of tricalcium phosphate in original culture
Amount of alkali in cc

FIG. 2. TITRATION CURVES OF THE CULTURE OF *Thibacillus thiooxidans* N. SP.

The sulfur is oxidized into sulfuric acid; this acid acts upon the tri-calcium phosphate transforming it first into the di-calcium salt, then the mono-calcium salt, and finally into phosphoric acid, while the calcium is precipitated as calcium sulfate; further oxidation of sulfur results in the production of free sulfuric acid.

As to the influence of initial reaction upon growth, we find that a reaction having a hydrogen-ion concentration equivalent to a pH of 2.0–2.8 is the most favorable for the growth of the

organism. Reactions more acid than 2.0 easily become injurious, although the organisms still continue to live at even as low a reaction as a hydrogen-ion concentration of pH = 0.6, while the medium titrates 0.8 normal acid (with phenolphthalein as indicator, using $\frac{N}{10}$ NaOH: the culture being grown on medium 1). Reactions ranging in pH from 4.0 to 6.0 are less favorable. Growth is slower to start, but once the reaction, through a slow oxidation of the sulfur, has reached a pH of about 3.0, the growth becomes more rapid. Reactions equivalent to pH 6.0 and above are unfavorable for growth. When a culture, at a pH 0.8 to 1.6 (these being the limits tested), is filtered free from any unoxidized sulfur, then stoppered and allowed to stand, the liquid is found to clear up, after a period of time, and the bacteria are agglutinated with the formation of flaky masses at the bottom of the containers. The rapidity of agglutination depends on the reaction of the culture, the more acid cultures agglutinating more rapidly than the less acid ones: at a pH = 0.8, agglutination took place in four to five days, while at pH = 1.5, it took more than two weeks for this phenomenon to appear. It is interesting to note that this phenomenon was never observed in the unfiltered culture, i.e., in the presence of unoxidized sulfur, even if the cultures were kept at pH 0.6 to 0.8 for a long time.

Neutralizing agents. The acid formed rapidly changes the hydrogen-ion concentration of the medium and growth almost ceases. To obviate a rapid change in reaction by the acid produced from the oxidation of the sulfur, neutralizing agents are to be used. These should be of such a nature as not to make the medium alkaline or tend to change the reaction rapidly: this eliminates, therefore, the use of carbonates and soluble oxides, like CaO. The best substances are buffers, like phosphates, but to keep the reaction above a very high acidity by means of soluble phosphates, high concentrations have to be used, which will exert an unfavorable physical effect upon the organism. Solid salts, insoluble in water which, on dissolving by the action of the acid, will give a soluble substance and an insoluble residue, are best for this purpose. $CaCO_3$ and $MgCO_3$ can be used, but

these go rapidly into solution by the action of the acid, thus tending to change the reaction towards alkaline. $Ca_3(PO_4)_2$ offers the best material for the purpose, because, on dissolving, it gives an acid salt and an insoluble residue ($CaSO \cdot 2H_2O$).

Mechanism of sulfur oxidation. The sulfur is oxidized, according to the following reaction:

$$S_2 + 3O_2 + 2H_2O = 2H_2SO_4 + 282 \text{ Cal.} \quad (1)$$

In the presence of tri-calcium phosphate:

$$Ca_3(PO_4)_2 + 2H_2SO_4 = Ca(H_2PO_4)_2 + 2\,CaSO_4 \quad (2)$$

$$Ca(H_2PO_4)_2 + H_2SO_4 = 2\,H_3PO_4 + CaSO_4. \quad (3)$$

The energy liberated in the oxidation of sulfur is used by the organism for its activities. The acid formed interacts with the neutralizing agents of the medium, giving first mono-calcium phosphate, at a pH of about 2.8–3.0, then phosphoric acid. So that, at a condition of equilibrium, we have a mixture of phosphoric and sulfuric acids, and the calcium salts of these acids, the condition of equilibrium depending on the stage of oxidation.

Taxonomic considerations. The first paper of this series contains a study of the five groups of sulfur bacteria. The organism described in this paper, *Thiobacillus thiooxidans* n. sp., is placed in a fifth group, which includes members morphologically related to the members of the fourth group, but which are distinctly different physiologically.

Group four includes colorless sulfur bacteria which do not accumulate sulfur within their cells, but which produce an abundance of sulfur (from H_2S and thiosulfates) outside of their cells. This group of bacteria is the one closely related to the organism studied in this paper and will, therefore, be discussed in greater detail. Group four is represented by two bacteria, *Thiobacillus thioparus* (Nathanson) Beijerinck and *Thiobacillus denitrificans* Beijerinck. Group five, which is so far represented only by *Thiobacillus thiooxidans* n. sp., will include colorless sulfur-oxidizing bacteria which do not accumulate sulfur either

FIG. 3. *Thiobacillus thiooxidans*, N. SP. (× 1500). CULTURE GROWN IN INORGANIC MEDIUM

Stained with aqueous-alcoholic solution of gentian violet

within or without their cells, which are very small in size (a micron or less in length and 0.5 micron in diameter) and which oxidize sulfur rapidly to sulfuric acid with a very acid reaction.

Thiobacillus thioparus was demonstrated by Nathanson in sea water and by Beijerinck in canal water. It was isolated on a medium containing sodium thiosulfate as a source of sulfur, in addition to minerals and ammonium chloride (0.01 per cent) and sodium bicarbonate as a source of carbon. In two to three days, the surface of the medium became covered with free sulfur filled with bacteria. This organism is 3 by 0.5μ, not forming any spores, is very motile and very sensitive, dying out on the plate in a week. The thiosulfates can be replaced by CaS, H_2S and elementary sulfur.

Jacobsen dissolved the sulfur in sodium sulfide and precipitated with dilute hydrochloric acid, washed and dried, then added to a medium containing 100 parts of water, 0.05 K_2HPO_4, 0.05NH_4Cl, 0.02 $MgCl_2$, 2 of $CaCO_3$ or $MgCO_3$ and a trace of $FeCl_3$ (3 parts of NaCl were used in the case of the organism isolated from sea water). The cultures were incubated at 30°C. The organism was found to form a film on the surface of the culture, with sulfur granules surrounding the cells; at the end, instead of sulfur, only a slimy bacterial mass was found to remain. Traces of hydrogen sulfide were always found. Pure cultures of the organism were obtained on agar plates, using 0.5 per cent of sodium thiosulfate and some $CaCO_3$. The carbon dioxide is obtained from carbonates, no growth being obtained and no sulfuric acid produced in the absence of carbonates. The organism is autotrophic since it does not require any organic matter for its development; it is sometimes motile and sometimes nonmotile.

Thiobacillus denitrificans was isolated by Beijerinck by adding to 100 parts of canal water, 10 parts of powdered sulfur, 0.05 KNO_3, 0.02Na_2CO_3, 2$CaCO_3$, 0.02K_2HPO_4 and 0.01 part of $MgCl_2$, and incubating the medium at 30°C. The sulfur was oxidized and growth was accompanied by a reduction of the nitrate to atmospheric nitrogen. The organism was isolated on agar plates and was found to be a motile, short rod, hardly distinguishable

morphologically from the *Thiobacillus thioparus*. Both organisms use carbonates and bicarbonates as sources of carbon and rapidly lose, on the plate, their ability to grow.

The following table gives the salient features of organisms belonging to groups 4 and 5.

Autotrophy. *Thiobacillus thiooxidans* belongs to the autotrophic bacteria which derive their energy from inorganic substances,

TABLE 4

Salient features of sulfur oxidizing bacteria, not accumulating sulfur within their cells

	TH. THIOPARUS (NATHANSON) BEIJERINCK	TH. DENITRIFICANS BEIJERINCK	TH. THIOOXIDANS N. SP.
Energy	H_2S, thiosulfate, sulfur	H_2S, thiosulfate, sulfur	Sulfur, thiosulfate
Size	3 by 0.5 μ	3 by 0.5 μ	1 by 0.5 μ
Accumulation of sulfur outside the cell	+++	+++	None
Pellicle formation	+	+	None
Carbon sources	Carbonates, bicarbonates	Carbonates, bicarbonates	CO_2 from atmosphere
Aerobism	Aerobic	Anaerobic	Aerobic
Growth on agar media	+	+	−
Motility	+	+	±
Acid accumulation	Active	?	Very strong, pH goes down to 0.6

and its carbon from the CO_2 of the atmosphere. This bacterium, which can derive its carbon from the CO_2 of the atmosphere, its energy from inorganic sulfur, its nitrogen from ammonium sulfate and other inorganic salts and whose mineral need is very small, was probably among the very first to start life on our planet. The sulfuric acid formed interacted with the insoluble silicates, phosphates, carbonates, etc., thus helping to break down the original rock and allowing the life of other organisms to follow. This organism or, perhaps group of organisms, together with the nitrifying bacteria may thus have formed the initial step in the organic world, manufacturing organic materials for other forms of life to follow.

SUMMARY

1. *Thiobacillus thiooxidans* n. sp. was isolated from composts of soil, sulfur and rock phosphate, by the use of inorganic media.

2. It oxidizes elementary sulfur to sulfuric acid, derives the necessary carbon from the CO_2 of the atmosphere and its nitrogen need from inorganic nitrogen salts.

3. It is responsible for the oxidation of sulfur in the soil and when soil is composted with sulfur or with sulfur and rock phosphate.

4. The sulfuric acid produced from the oxidation of sulfur by *Thiobacillus thiooxidans* n. sp. transforms tri-calcium phosphate into soluble phosphates and finally into phosphoric acid.

5. *Thiobacillus thiooxidans* n. sp. produces more acid, from oxidation of sulfur, and continues to live in a more acid medium, than any other living organism yet reported, the hydrogen-ion concentration of the medium increasing to a pH 0.6 and less.

REFERENCES

BEIJERINCK, M. W. 1904 Über die Bakterien, welche sich im Dunkeln mit Kohlensäure als Kohlenstoffquelle ernähren konnen. Centralbl. f. Bakt., 2 Abt., **11**, 593–599.

COHN, F. 1875 Untersuchungen über Bakterien, II, Beitr. Biol. Pflanz., **1**, H. 3, 141.

CORSINI, A. 1895 Über die sogenannten "Schwefelkörnchen" die man bei der Familie der Beggiatoaceae antrifft. Centralbl. f. Bakt., 2 Abt, **14**, 272–289.

CRAMER, In Müller, C. 1870 Chemischphysikalische Beschreibung der Thermen von Boden in der Schweiz.

DÜGGELI, M. 1917 Die Schwefelbakterien und ihre Tätigkeit in der Natur. Natur wiss. Wochschr., **16**, No. 24, 321–328.

DÜGGELI, M. 1919 Die Schwefelbakterien. Neujarsblatt d. naturf. Gesell. Zürich. No. 121, 43p.

GEHRING, A. 1915 Beiträge zur Kenntnis der Physiologie und Verbreitung denitrifizierender Thiosulfat-Bakterien. Centralbl. f. Bakt., 2 Abt., **42**, 402–438.

GICKLEHORN, J. 1920 Über neue farblose Schwefelbakterien. Centralbl. f. Bakt., 2 Abt., **50**, 415–427.

HINZE, G. 1903 *Thiophysa volutans*, ein neues Schwefelbakterium. Ber. deut. bot. Gesell, **21**, 309–316.

HINZE, G. 1913 Beiträge zur Kenntniss der farblosen Schwefelbakterien. Ber. deut. bot. Gesell., **31**, 189–202.

HEUPPE, F. 1905 Über Assimilation der Kohlensaure durch chlorophyllfreie Organismen. Arch. Anat. u. Physiol., Physiol. Abstr., 33.

JACOBSEN, H. C. 1912 Die Oxydation von elementarem Schwefel durch Bakterien. Folia microb., **1**, 487–496.

JACOBSEN, H. C. 1914 Die Oxydation von Schwefelwasserstoff durch Bakterien. Folia microb., **3**, 155–162.

JEGUNOW, M. 1896 Bakterien-Gesellschaften. Centralbl. f. Bakt., 2 Abt., **2**, 11–21, 441–449, 487–482, 739–752.

JEGUNOW, M. 1898 Platten der roten und der Schwefelbakterien. Centralbl. f. Bakt., 2 Abt., **4**, 257.

KEIL, F. 1912 Beiträge zur Physiologie der farblosen Schwefelbakterien. Beitr. Biol. Pflanz., II, 335–302.

KOLKWITZ, R. 1912 Über die Schwefelbakterien *Thioploca ingrica* Wislouch. Ber. deut. bot. Gesell., **30**, 662–666.

KRUSE, W. 1910 Allgemeine Mikrobiologie.

LIESKE, R. 1912 Untersuchungen über die Physiologie denitrifizierender Schwefelbakterien. Ber. deut. bot. Gesell., **30**, 12–22.

LIPMAN, J. G., WAKSMAN, S. A., AND JOFFE, J. S. 1921 The oxidation of sulfur by soil microörganisms. Soil Sci., **12**, 475–489.

LOCKETT, M. T. 1914 Oxidation of thiosulfate by certain bacteria in pure culture. Proc. Roy. Soc., **87**, 441.

MCLEAN, H. C. 1918 The oxidation of sulfur by microörganisms in its relation to the availability of phosphates. Soil Sci., **5**, 251–290.

MEYERHOF, O. 1916 Untersuchungen über den Atmungsvorgang nitrifizierender Bakterien. Arch. Ges. Physiol., **164**, 353–427; **165**, 229–284.

MOLISCH, H. 1907 Die Purpurbakterien nach neuen Untersuchungen. Jena.

MOLISCH, H. 1912 Neue farblose Schwefelbakterien. Centralbl. f. Bakt., 2 Abt., **33**, 55–62.

NADSON, G. A. 1903 On the sulfur microörganisms in the Gulf of Hapsala. Bull. jard. Bot. St. Petersburg., **13**, 102–112.

NATHANSON, A. 1903 Über eine neue Gruppe von farblosen Schwefelbakterien und ihren Stoffwechsel. Mitt. zool. Station, Neapel. **15**, 655.

OMELIANSKI, W. 1913 Der Kreislauf des Schwefels. Lafar's Handb. d. techn. Mykol., **3**, 214-244.

OMELIANSKI, W. 1905 Über eine neue Art farbloser Thiospirillen. Centralbl. f. Bakt., 2 Abt., **14**, 769–772.

SAWJALOW, W. 1913 Über die Schwefelwasserstoffgärung im Schwarzen Heilschlamm. Centralbl. f. Bakt., 2 Abt., **39**, 440–447.

WAKSMAN, S. A., AND JOFFE, J. S. 1921 The oxidation of sulfur by microorganisms. Proc. Soc. Exp. Biol. Med., **18**, 1–3.

WAKSMAN, S. A., AND JOFFE, J. S. 1921 Acid production by a new sulfur oxidizing bacterium. Science, N. S., **53**, 216.

WEST, G. S., AND GRIFFITH, B. M. 1913 The lime-sulfur bacteria of the genus Hillomia. Ann. Bot., **27**, 83–91.

WINOGRADSKY, S. 1887 Über Schwefelbakterien. Bot. Ztg., **45**, No. 31–37

WINOGRADSKY, S. 1888 Beiträge zur Morphologie und Physiologie der Bakterien. I. Schwefelbakterien. Leipzig. 1–120.

WISLOUCH, S. M. 1912 *Thioploca ingrica* nov. spez. Ber. deut. bot. Gesell., **30**, 470–473.

THE PRODUCTION OF PINK SAUERKRAUT BY YEASTS[1]

E. B. FRED AND W. H. PETERSON

From the Departments of Agricultural Bacteriology and Agricultural Chemistry, University of Wisconsin, Madison

Received for publication June 25, 1921

Sauerkraut, or sour cabbage as it is sometimes called, is obtained by the acid fermentation of cabbage. The process of fermentation and manufacture is simple and the resulting product is greatly relished by many people. Some idea of the importance of this method of preserving cabbage may be seen from a glance at the sauerkraut industry in Wisconsin. In this state alone more than 36,000,000 pounds of sauerkraut are manufactured annually, in addition to that prepared in small quantities in innumerable households.

Normal sauerkraut has a distinctly acid reaction and a faint pleasant aroma. The shredded cabbage after it has turned into kraut loses some of its toughness, but should still retain a comparatively firm texture; the white color tends to lose its opaqueness and the cabbage becomes slightly translucent. Kraut with a strong odor and soft texture is of poor quality.

The preservation of cabbage in the form of sauerkraut is generally a result of natural fermentation. Clean white cabbage is cut into shreds, salt is added, and the entire mass packed into a vat and heavy weights are placed on top. In a few hours fermentation begins and the sugars of the cabbage are rapidly converted into lactic acid, acetic acid, alcohol, and small amounts of other products. Although many kinds of microörganisms may be found in the juice of the kraut, the lactic acid bacteria are the most important.

[1] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

In recent years, some of the canning companies have found it difficult always to secure a sauerkraut of good flavor, texture, and color. One of the difficulties encountered, has been the occurrence of a sauerkraut with a well defined pink or salmon-pink color. Although not unfit for food, this pink colored sauerkraut is an undesirable product as it must be sold at a price lower than that obtained for white kraut.

From a review of the literature, it seems probable that microorganisms are involved in the formation of the pigment. In 1904 Butjagin, and a year later, Wehmer called attention to the occurrence of pink producing organisms in sauerkraut. Henneberg in 1916 reported that the addition of 1.2 per cent of lactic acid to cabbage resulted in the production of a reddish colored kraut. The presence of large numbers of yeasts in sauerkraut, and in a few cases of pink yeasts, has been reported by various investigators. The distribution of these pink yeasts in nature and the factors that influence pigment formation have been the source of much study. Grösbusch (1915) isolated from apples a colorless torula which under certain conditions formed a deep red pigment. Some of the conditions which he found favorable for pigment production, were low sugar content, certain kinds of sugars, and a slightly acid reaction. Beijerinck (1919) decribed a yeast producing a colorless substance which became a deep red pigment in the presence of oxygen and iron salts. A complete discussion of the literature of the pink yeasts will be found in the papers of Will (1907; 1912), Pringsheim and Bilewsky (1911). In view of the occurrence in practise of the colored sauerkraut it becomes a matter of some importance to discover the cause, and if possible, the remedy for this undesirable type of fermentation.

EXPERIMENTAL

Large samples of normal sauerkraut and pink sauerkraut were secured from one of the canning companies and analyzed. This kraut was six weeks old and judged proper for canning. The comparative chemical and bacteriological analyses are given below:

Analysis of sauerkraut

	NORMAL	PINK
Chemical:		
1. Per cent of water in kraut	90.6	88.0
2. Total titratrable acid in 100 cc. juice	140.0 cc. 0.1N	147.5 cc. 0.1N
3. Volatile acid as acetic in 100 cc. juice	0.247 gram	0.255 gram
4. Non-volatile acid as lactic in 100 cc. juice	1.026 grams	1.426 grams
5. Alcohol as ethyl	0.727 gram	0.978 gram
Bacteriological:		
Number of microörganisms in 1 cc. juice	3,600,000	91,000,000

Chemical analysis failed to show any striking difference in composition between the normal and the pink sauerkraut. Later analyses of other samples indicate that the figures given above are fairly representative of the two kinds of sauerkraut. The results of the bacteriological analyses are of more significance and show that the colored kraut is much richer in microörganisms. By means of direct microscopic mounts from the normal kraut it was found that there was a preponderance of rod-formed bacteria while the pink kraut contained yeast cells almost exclusively. The enormous number of yeast cells in the juice of pink kraut suggested that these organisms might be the cause of the pink pigment. Prompted by the fact that yeasts are usually present in high numbers in pink sauerkraut a great number of dilution plates were poured. Almost without exception the colonies on these plates consisted of yeasts[2] but rarely was any pigment developed.

Many samples of pink sauerkraut were plated and from well isolated colonies transfers were made to glucose yeast-water agar slants. In general these yeasts from pink kraut showed little if any color on agar slants. A few transfers gave a pale pink color. From a large number of cultures three pink colored colonies were selected for further study, numbered 24–1, 85–1, and 95–6. These strains show a difference in color; 24.1 and

[2] The term yeasts in this paper is used to designate the true *saccharomycetes* and also those which do not form ascospores, the *torulae*. It is probable from the results of previous workers that these pink yeasts are properly termed *torulae*.

95.6 are a salmon shade of pink and 85.1 is a magenta shade. In form these organisms vary from round to oval, but elongated cells were the most abundant.

Factors that influence color

Oxygen. The influence of oxygen on the production of pigment by these yeasts was determined by growing the cultures in the air, and in a desiccator where only a limited supply of air was available. In the presence of air the growth and pigment production was good while in its absence a fair growth and no color was obtained. When these colorless tubes were exposed to the atmosphere a salmon-pink color developed rapidly usually within one to two days. The influence of oxygen was also noted when kraut was exposed to the air, e.g., pale pink kraut turned a deeper color after a few minutes exposure.

Reaction. The reaction of the medium for cultivating these organisms was varied between the pH values of 6.5 and 7.2. Pigment was formed in all cases and approximately to the same extent. At a reaction of 5.5 the growth was not so rapid, but the color was somewhat more brilliant.

Temperature. The influence of this agent on color formation by these yeasts was studied at 18°, 22°, 28°, and 37°C. Only a very scanty growth was noted at 37°C, a profuse growth at 22° and 28°C, and a fair growth at 18°C. The deepest pigment was found in the tube cultures at 22°C. and next in intensity of color, at 28°C. Although the higher temperature of 28°C. resulted in a profuse growth the pink color was not nearly so noticeable as at lower temperatures. Apparently about 22°C. or 71°F. gave the most marked color.

Iron and manganese salts. The influence of iron citrate, iron ammonium citrate, iron lactate, iron sulphates, and manganese sulphate, on pigment formation was studied. A decided difference in behavior of the various strains of yeast towards the iron and manganese compounds was noted. Strain 85–1 responded far more to the iron salts than either strain 24–1, or 95–6. Of the various compounds, iron lactate proved the best stimulant forpigment production. The other compounds, e.g.,

iron citrate and iron ammonium citrate also favored pigment production. The manganese salts apparently promoted growth but did not bring about an increase in the pigment formation.

Sugars. In order to overcome as much as possible the breaking down of the sugars from the high heat, concentrated solutions of sugars in water were sterilized and added to the culture medium when cool. The yeast medium contained the following constituents:

Ammonium sulphate	3.0 grams
Asparagin	1.5 grams
Dibasic potassium phosphate	2.0 grams
Calcium chloride	0.25 gram
Magnesium sulphate	0.25 gram
Sugar	20.0 grams
Agar	15.0 grams
Water	1000.0 cc.

Three sugars were studied; xylose, glucose and maltose in 2 per cent solutions. Each strain of yeast was grown in triplicate on agar slants, containing each sugar, and these cultures were incubated at 20° to 22°C. At regular intervals, usually of 1 week each, these cultures were examined for rate of growth and pigment production.

All three strains showed by far the most rapid growth in the glucose medium, with maltose next in order, and xylose last. The decided difference between growth and pigment formation is brought out in a striking manner from the results of this test. Without exception the easily fermentable sugar, glucose, gave a profuse growth and only a trace of pigment. Somewhat similar results were obtained with maltose although a pale pink was noted. In the presence of xylose these yeasts grew slowly but produced a decided pink color. From these results it is clear that the sugar, xylose, which is fermented only with difficulty is the best one of the three for pigment production. After thirty days the glucose cultures entirely lost their color while the other cultures became a deeper pink. Our results agree with those of Grösbusch, who found that the best color was obtained in the case of the non-fermentable sugars, arabinose and raffinose.

TABLE 1

Effect of sodium chloride on pigment production

NUMBER	TIME	SODIUM CHLORIDE	XYLOSE		GLUCOSE		MALTOSE	
			Growth	Color	Growth	Color	Growth	Color
			Cultures 24–1					
	days	*per cent*						
1	8	None	Good	Pink	Profuse	Pink	Profuse	Pink
2	8	2	Fair	Pink	Profuse	Pink	Profuse	Pink
3	8	4	Trace	Pale pink	Fair	Pink	Moderate	Pink
4	30	None	Profuse	Pink	Profuse	Pale pink†	Profuse	Pink
5	30	2	Profuse	Pink	Profuse	Pink	Profuse	Brilliant pink
6	30	4	Moderate	Brilliant pink	Profuse	Pink	Profuse	Brilliant pink
			Cultures 85–1					
7	8	None	Scanty	No color	Scanty	Pale pink	Scanty	No color
8	8	2	Scanty	No color	Scanty	Pale pink	Scanty	No color
9	8	4	Scanty	No color	Scanty	Pale pink	Scanty	No color
10	30	None	Moderate	Pink*	Fair	Pale pink	Slight	Pale pink
11	30	2	Moderate	Pink	Fair	Pink	Slight	Pink
12	30	4	Moderate	Pink	Fair	Pink	Slight	Pink
			Cultures 95–6					
13	8	None	Scanty	Pink	Scanty	No color	Scanty	Pink
14	8	2	Scanty	Pale pink	Scanty	No color	Scanty	Pale pink
15	8	4	Scanty	Pale pink	Scanty	No color	Scanty	Pale pink
16	30	None	Fair	Pink	Scanty	No color	Fair	Pale pink
17	30	2	Fair	Pink	Scanty	No color	Fair	Pink
18	30	4	Fair	Pale pink	Scanty	No color	Fair	Pink

* Deeper pink than any other sugar.

† Beginning to lose color.

Sodium chloride. The influence of salt on pigment production naturally suggested itself since it has been reported by many experienced makers of sauerkraut, that high concentrations of salt caused the reddening of the kraut. Sodium chloride in concentration of 2 and 4 per cent was added to triplicate tubes of the glucose, maltose and xylose agar media. These tubes were inoculated with each yeast. 2.5 per cent salt is the approximate amount used in the manufacture of commercial kraut. In table 1 are given a summary of the results of these tests. The cultures plus salt developed much more slowly at first than those without salt, but after two weeks this retardation was not so noticeable. In the glucose series, culture 24–1, no salt, the pink color began to fade to a pale pink at the end of two weeks, while in the presence of salt a brilliant pink pigment persisted. These cultures were kept for 6 weeks but without any loss of color, save in the no-salt group. Instead of a loss, the older cultures of the salt group showed a greater amount of pigment and a deeper color. Somewhat similar results were secured with the other strains of yeasts although the intensifying effect of salt was not so noticeable. It seems safe to conclude that sodium chloride even in large amounts exerts a favorable effect on pigment production. This favorable influence in not noticeable until the cultures are several weeks old.

PRODUCTION OF PINK SAUERKRAUT BY INOCULATION WITH YEASTS

In large glass percolators of 2 liter capacity 1000 grams of cut cabbage were packed. The outside leaves and core of the raw cabbage were removed, it was cut on a small shredder and salt was added. Part of the cabbage was inoculated with cultures of the yeast isolated from pink kraut. The entire mass was packed into the percolator and weighted down with a bottle of sand or mercury, that weighed 1 kilo and which fitted closely in the top of the percolator. The lower end of the percolator was fitted with glass wool, and below this was inserted a one hole rubber stopper fitted with a glass tube. Through this glass tube, which was sealed at one end with a rubber tube and screw clamp,

† Beginning to lose color.

* Deeper pink than any other sugar.

samples of the fermenting liquid were removed from time to time and the total titratable acidity measured. For comparison, samples were also taken from the top of the percolators and titrated. No very decided difference was noted in titration figures from the different parts of the same container.

Since it is highly important to note the color changes during fermentation, percolators were found especially suitable for this study.

The plan of this experiment follows:

1. 2 per cent salt no inoculation,.....Control
2. 2 per cent salt no inoculation,.....Control
3. 2 per cent salt inoculated with yeast.....24–1
4. 4 per cent salt inoculated with yeast.....24–1
5. 2 per cent salt inoculated with yeast.....85–1

After four days the cabbage in percolators, numbers 3, 4, and 5 showed numerous pink colored spots and two days later the the entire mass became pink throughout. The pink pigment of numbers 3 and 4 especially increased with age, until at the end of two weeks the plant tissue was a deep red or a purplish red color. This increase in color as the kraut aged, was not noted in the cabbage inoculated with 85–1. When four weeks old the kraut was removed and examined.

Numbers 1 and 2 were sour, rather tough and judged of fair quality. Direct microscopic mounts showed a preponderance of bacteria.

Numbers 3 and 4 were bitter with an unpleasant flavor. Microscopic mounts showed an almost equal number of yeasts and bacteria.

Total acid determinations, made at two-day intervals failed to bring out any very striking differences; in general the containers with yeasts showed less acid at the end of the experiment than the untreated controls. The total titratable acidity at the time of opening is given below:

	0.1 N acid in 100 cc. of juice cc.
1. Control	218.0
2. Control	162.0
3. Inoculated with 24–1	73.0
4. Inoculated with 24–1	144.0
5. Inoculated with 85–1	179.0

EFFECT OF SODIUM CHLORIDE AND OF TEMPERATURE ON THE PRODUCTION OF PINK SAUERKRAUT

Attention has been called to the statement of sauerkraut manufacturers that salt favors the pink color. Laboratory tests with pure cultures of pink yeasts also indicate the favorable

TABLE 2

The influence of sodium chloride and temperature on the production of pink sauerkraut

AGE OF KRAUT	SODIUM CHLORIDE	TEMPERATURE IN CENTIGRADE		
		28°	21°	16°
days	*per cent*			
7	2.5	A trace of pink	No pink color	No pink color
7	3.5	A trace of pink	No pink color	No pink color
7	4.5	Pink at bottom	No pink color	No pink color
7	5.5	Brilliant pink throughout	No pink color	No pink color
10	2.5	Spots of pale pink	No pink color	No pink color
10	3.5	More pink than above	No pink color	No pink color
10	4.5	Pink at bottom	No pink color	No pink color
10	5.5	Brilliant pink throughout	No pink color	No pink color
20	2.5	Pale pink	No pink color	No pink color
20	3.5	Pink	Pale pink	No pink color
20	4.5	Brilliant pink throughout	Pink spots	No pink color
20	5.5	Brilliant pink throughout	Pink throughout	Pink spots

influence of this chemical on color production. To see if the raw cabbage without inoculation, but high in salt, would undergo a fermentation which produced a pink color, 12 large percolators of cabbage were treated as outlined in the table below and incubated at three temperatures, 16°, 21°, and 28°C. The procedure

was the same as in the previous experiment. Table 2 gives the general plan and results of this test. The data recorded in the table show: first, the close relation between amount of sodium chloride and formation of pink sauerkraut; second, the decided influence of temperature on color. These two points are so clearly brought out in the table that a detailed explanation is not needed. The findings are in accord with the observations of kraut manufacturers; namely, that high salt concentration may cause pink sauerkraut.

TABLE 3

Effect of sodium chloride on the production of acids by lactic acid bacteria; calculated for 100 cc. of culture

NUMBER	MEDIUM	TIME AFTER	SODIUM CHLORIDE	0.1N ACID
		days	*grams*	*cc.*
1	Cabbage juice	3	None	70.4
2	Cabbage juice	3	1	55.6
3	Cabbage juice	3	2	49.8
4	Cabbage juice	3	3	32.2
5	Cabbage juice	3	4	1.8
6	Cabbage juice	3	5	1.2
7	Cabbage juice	3	6	0.0

An explanation for this condition may be found from a study of pure cultures of yeasts and bacteria common to sauerkraut. Yeasts can live in the presence of high concentrations of salt while the bacteria commonly associated with kraut are not so resistant. Support for this statement is given in the figures of table 3. Here the influence of varying amounts of sodium chloride on a pure culture of lactic acid bacteria was studied. This lactic acid organism was isolated from normal sauerkraut. About 4 per cent of salt is the critical concentration for this organism.

Orla-Jensen (1919) has carried out an extensive study of the influence of sodium chloride on acid production by various types of lactic acid bacteria. He found that the common forms of lactic acid bacteria, the *Bacterium lactis-acidi*, *Lactobacillus bulgaricus*, and other types are slightly retarded by 2.5 per cent of salt. On the other hand, certain of the rod forms of lactic

acid bacteria isolated from plant tissue are not retarded in the least by this concentration of salt. In the presence of larger amounts of salt (5.5 per cent), all of the rod forms of lactic bacteria are injured. A concentration of not more than 2 to 4 per cent of salt would not seriously retard the growth of the yeast and would favor its production of pigment.

EFFECT OF HIGH ACID FORMING BACTERIA ON THE PRODUCTION OF PINK SAUERKRAUT

The use of bacteria which would ferment the sugars of the cabbage juice rapidly naturally suggests itself as a means of combating the growth of pink yeasts. If great numbers of acid producing bacteria are seeded in the raw cabbage then it is possible that they will so dominate the fermentation that the wild yeasts will not be able to exert any well defined effect on the kraut. The fallacy of this assumption, however, is clearly seen from the results of table 4. As in the preceding tests large percolators with 1000 grams each of raw cabbage were used. The data include the results of four separate tests carried out at different times. In every case before the end of the fermentation period the cabbage to which acid was added or which was inoculated with high acid-producing bacteria turned pink. Apparently the high acid production which followed inoculation favored the growth of pink yeasts. Direct microscopic mounts made from this pink sauerkraut furnished support for this statement When opened, the inoculated kraut showed great numbers of yeasts. Some idea of the rate of acid formation may be gained from the figures in this table. In the early stages of fermentation, without exception, the kraut seeded with aciduric bacteria showed a much greater quantity of acid than the control kraut. This difference is especially noticeable in the titration figures of the first four or five days. After eight days, conditions changed and before the end of the fermentation the control krauts were far more acid than the inoculated krauts. Culture 124-1 which is a high acid former from pentoses and other sugars is especially favorable for the development of the pink yeasts. On the other hand the non-pentose fermenter *Bact. lactis-acidi* which

produces acid more slowly than Culture 124–1, does not favor the growth of the pink yeasts. Perhaps the beneficial effect of inoculation on pigment production is due to the rapid destruction of the easily fermentable sugars and to the change in reaction. This combination of conditions no doubt reduces greatly the complex of the bacterial life and thus favors the development of the wild yeasts.

TABLE 4

The relation of acid forming bacteria and pink yeast to the production of pink sauerkraut

NUMBER	TREATMENT	0.1N ACID IN 100 CC. OF JUICE				NOTES	
		Age in days					
		2	4	8	20	Quality	Appearance
		cc.	cc.	cc.	cc.		
1	Control	28	79	195	192	Fair	White
2	*Lactob. bulgaricus*	83	136	156	155	Poor	Pink
3	Control	23	99	165	218	Raw	Trace of pink
4	Culture 52–7	92	120	122	169	Fair	Pale pink
5	Culture 124–1	111	150	168	152	Raw	Brilliant pink
6	Culture 124–1	114	150		161	Raw	Pink
7	Control	25	79	121	197	Good	White
8	Culture 124–1	95	131	145	153	Poor	Pink
9	Culture 124–1	84	135	140	151	Poor	Pink
10	Control + acids*	110	121	125	192	Poor	Deep pink
11	Culture 85–1	23		43	114	Fair	White
12	Culture 85–1 + acids*	110		130	173	Raw	Deep pink
13	Culture 85–1 and 124–1	39		115	118	Poor	Pink
14	Culture 24–1 and *Bact. lactis acidi*	31		96	142	Good	White

* Acetic and lactic acids equivalent to 109 cc. 0.1N per 100 cc. of juice.

The stimulating effect of acids on pigment production is further indicated by the results obtained in containers Nos. 10 and 12 where acetic and lactic acids were added to uninoculated cabbage and to cabbage inoculated with yeasts. The quantity of acid added was approximately that found in a moderately ripened kraut. Pigment was observed after seven days which is about the earliest appearance of color in any of the experiments.

SUMMARY

The pink or red color of sauerkraut is due to the growth of certain yeasts or torulae. Although these organisms are commonly found in great numbers in kraut, they frequently fail to show any pigment. The production of pigment is not a fixed characteristic but depends upon many factors. The following conditions influence the formation of color: kind of sugar, amount of sugar, amount of sodium chloride, reaction, temperature, and oxygen supply. The chief factors in the production of pink sauerkraut are high temperature, high salt concentration and high acid content. When cabbage is allowed to ferment at a temperature of 20°C. or above a pink pigment is frequently noted. Almost without exception, cabbage fermented in the presence of large amounts of sodium chloride, 3 per cent or more, showed a decided pink or red color. High acid producing bacteria as well as the direct addition of acids to cabbage favor the development of pink kraut.

REFERENCES

ADERHOLD, R. 1906 Sauerkraut. Lafar's Handbuch d. Tech. Mykologie, **2**, 319–322.

BEIJERINCK, M. W. 1919 Chromogenic yeasts. Chem. Absts., **13**, 1082.

BUTJAGIN, B. 1904 Vorläufige Mitteilung über Sauerkrautgärung. Centralbl. f. Bakteriol., II, **11**, 540.

GRÖSBUSCH, T. 1915 Über eine farblose stark roten Farbstoff erzeugende Torula. Centralbl. f. Bakteriol., II, **42**, 625–638.

HENNEBERG, W. 1916 Das Sauerkraut (Sauerkohl) Die deutsche Essigindustrie, **20**, No. 28, 194.

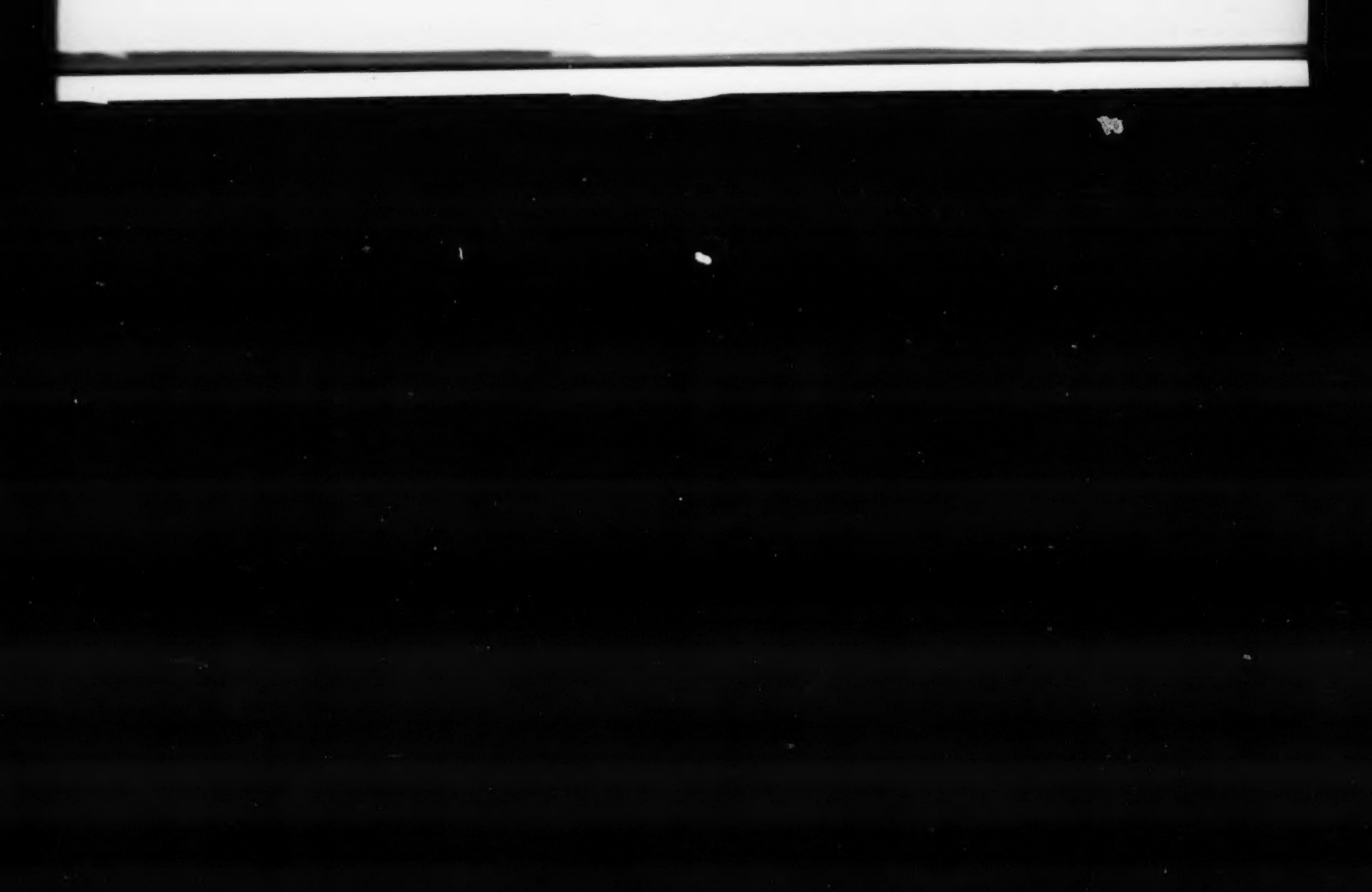
ORLA-JENSEN, S. 1919 The Lactic Acid Bacteria. Mémoires de l' Academie Royale des Sciences et des Lettres de Danemark, Copenhague. Section des Sciences, 8me serie, t. V, no. 2, pp. 81–196, with 51 plates.

PRINGSHEIM, E., AND BILEWSKY, H. 1911 Über Rosahefe. Beiträge z. Biologie der Pflanzen, **10**, 118–132.

WEHMER, C. 1905 Untersuchungen über Sauerkrautgärung. Centralbl. f. Bakteriol, II, **14**, 682–713; 781–800.

WILL, H. 1907 Rosahefen. Lafar's Handbuch d. Tech. Mykologie, **4**, 280–302.

WILL, H. 1912 Beiträge zur Kenntnis rotgefarbter niederer Pilze. Centralbl. f. Bakteriol., II, **35**, 81–118.



STUDIES ON THE BIOLOGY OF LACTIC ACID BACTERIA: A SUMMARY OF PERSONAL INVESTIGATIONS[1]

COSTANTINO GORINI

Bacteriological Laboratory of the Royal Superior School of Agriculture, Milan, Italy

Received for publication July 30, 1921

In the following pages are summarized the most important results of investigations which I have pursued during the recent war period upon the biological characteristics of the lactic acid bacteria.

ACIDO-PROTEOLYTIC PROPERTIES

One of the most important characteristics of many lactic acid bacteria is that of possessing acido-proteolytic properties. After the original discovery of this property I have often asserted that this activity must take place in natural milk of acid reaction, but that it is not observed in milk which has received the addition of chalk or other substances, since these additions not only alter the composition of the milk but also so alter its adaptability for the growth of the organisms concerned that the natural functions of the latter are no more exhibited and thus the results obtained in the study are no longer applicable directly to normal conditions such as occur in the cheese industry.

The detection of this acido-proteolytic property is very simple and indeed requires no chemical manipulations. Just as the liquefaction of gelatin indicates proteolytic properties, the casein cleaving properties of these organisms are indicated by the

[1] The investigations covered by this summary have been published in the transactions of the Reale Accademia dei Lincei of Rome and the transactions of the Reale Istituto Lombardo di Scienze e Lettere of Milan during the years 1914–1920.

liquefaction of the casein coagulum. I have described, as organisms possessing acido-rennin properties, cocci from the udder of the cow, and bacilli from cheese and fermented milk beverages such as Yoghurt and Gioddu. The inception of casein breakdown is dependent upon various circumstances; chief among these, and of paramount importance, is *temperature.*

In 1897 I demonstrated that while high temperatures are appropriate to the process of lactose fermentation, lower temperatures are more appropriate to the cleavage of casein, and in several additional publications I was able to verify my findings. Finally in 1915 I presented analytical data bearing upon the behavior of lactococci and lactobacilli at temperatures ranging between 25° and 35°C. and 15° to 20°C. Many lactic acid forming bacteria which failed to show lacto-proteolytic properties when cultivated at the higher temperatures were found to possess these properties when cultivated at the lower temperatures, *i.e.*, such temperatures as prevail during the process of cheese ripening.

Another condition of great importance for the furthering of the lacto-proteolytic activities is the *composition of the medium.* Already in 1902 I had advanced evidence as to the influence of the nature of the protein upon the activities of the organisms, indicating that while among the udder cocci some dissolved casein as well as gelatin, others only dissolved casein while again others were found that acted only on gelatin.

Another factor of great importance was found to lie in the *quality of the milk* itself, which presents great fluctuations the causes of which are to be found in the changes it undergoes previous to reaching the laboratory (besides the influence exerted by the race of the animals furnishing the milk, their physiological state, their feeding régime, etc.) or in the laboratory itself. In addition, in 1915, I was able to show the noxious influence exerted by the peptonized constituents of the milk derived from the casein cleavage. These constituents are often to be found in milk, especially in market milk, and are due to the great development of organisms before sterilization.

Another factor of great importance, and indeed of an essential character in the behavior of milk, is to be found in the *method of*

sterilization previous to use. Milk sterilized in the autoclave acquires a brownish tinge, indicative of a change of state, which makes it unfit for the demonstration of the proteolytic activities of the lactic acid bacteria. For such a study milk should be utilized that has been sterilized at a moderate heat so that its white color has been preserved. By using this white-sterile milk I was in a position to detect casein cleaving properties in some of the lactic acid bacteria which in brown-sterile milk failed to digest this compound. Some of the so-called propionic acid bacteria behaved similarly.

Undoubtedly, in this factor of appropriate sterilization is to be found the reason why many authors claim for the lactic acid bacteria negative or only negligible casein-acido-dissolving properties, and therefore contrary to my opinion have ascribed to them no rôle in the ripening of cheese.

BACTERIAL FLORA OF THE UDDER

The importance which the acid forming organisms seem to bear, both from the standpoint of hygiene and in the dairy, gave me occasion for a deep study. It is to be emphasized nevertheless that investigations seem to point to the fact that from the hygienic and sanitary standpoint it is not only the cleanliness of the stable and of the cow that are to be considered, but also other internal and external factors. A fact of great importance in this connection is, that apparently sound cows may harbor in their udder for longer or shorter periods of time bacteria (cocci as well as bacilli) which sometimes prove beneficial and other times noxious both from the hygienic as well as from the dairying standpoint. Therefore a selection of milch cows on the basis of this udder flora has been proved useful by me and to this I have given new support by carrying out fermentation tests as a means of judging of the quality of the milk, especially of such milk as is used for direct consumption by children and invalids.

HEAT RESISTANCE

It is generally assumed that an exposure of one quarter of an hour at 60° to 80°C. is sufficient to destroy the non-spore-forming

lactic bacteria. On the contrary I have discovered that the lethal temperature may reach 100°C. and sometimes still higher values.

My experiments have shown that this happens through the formation of a sheath of coagulated casein in immediate contact with the acid-rennin forming organisms. These findings allowed me to explain the apparent failures in milk sterilization, and they furnish the basis for a betterment of the processes in use for industrial milk sterilization.

VISCOSITY IN YOUNG CULTURES

Many lactic acid forming organisms are capable of inducing ropiness in milk only during the early phases of incubation. The property of making milk ropy, although recognized by various investigators for the lactic acid bacteria, has not generally been considered as a constant and essential character of this group of organisms. In 1912 I first described a lactic acid bacterium which constantly induced viscosity in milk, but only on the inception of acidification and before the inception of coagulation.

This organism at first induces ropiness, but the increase in acidity of the medium and the increase in the firmness of the coagulum soon obliterate this characteristic result of the growth. When cultivated at a not too high temperature and on white-sterile milk several other lactic organisms proved themselves, in my hands, to possess this property. The reason why many investigators disagree as to the constancy of this rope-producing property in many of the lactic acid bacteria is to be searched for in its transitory manifestation.

SPORE-PRODUCING LACTIC ACID BACTERIA

In 1904 I studied a spore-forming bacillus from cheese and described it under the name of *Bacillus acidificans-presamigenes-casei*, on account of its acid-rennet and acido-proteolytic characters and found it related to the subtilis or thyrotrix groups. In 1906 I found in silage another similar form and described and pictured it. Additional investigations have authorized me in the assertion that this type is rather widely disseminated in milk and dairy products and is therefore worthy of attentive study.

APPLICATION OF LACTIC ACID BACTERIA IN THE CHEESE INDUSTRY AND IN THE PREPARATION OF ACID SILAGE

In 1906 I first drew attention to the advantage to be derived from the utilization of the lactic acid bacteria in cheese manufacture, and in 1907 I recommended their utilization in the preparation of silage. Although from the beginning of my investigations I was fortunate in the selection of the appropriate types, so that their practical application now dates back fifteen years, my study was continued upon the various types which from time to time have attracted my attention. The result of these investigations is that several species, or varieties, of lactic acid bacteria can be utilized for the purpose, but that nevertheless it is not a matter of indifference, for the preservation of the character of determined cheese quality, which type has been used.

Two advantages are to be derived from the utilization of the lactic acid bacteria in cheese manufacture, *i.e.*, (1) the elimination of the noxious, putrefying and gas forming organisms and (2) the furthering of the appropriate ripening and an acceleration thereof. The first of the above aims is reached by the utilization of organisms that produce a high degree of acidity, whereas it is necessary for the attainment of the second aim that the organisms utilized possess the faculty of producing the appropriate products in the process of proteolysis. I will repeat here that the application of inoculation according to my method joins with the hygienic production and treatment of the milk in yielding a product practically free from undesirable microörganisms.

Similar considerations may be made with reference to the preparation of silage. Silage that has undergone the lactic acid fermentation is by far the best, both from the standpoint of cattle feeding as also from the standpoint of subsequent cheese manufacture. Every other silage is undesirable because, in spite of its satisfactory appearance and apparently low germ content, it is a carrier of butyric acid organisms.

The production of a lactic silage is best accomplished by following the accompanying general rules: (1) The use of siloes with impervious foundations; (2) a semi dried condition of the fodder;

(3) air exclusion from the mass obtained by the use of deep layers of silage and of strong packing whereby the temperature of the silage is kept between 35°C. and 40°C.; and (4) inoculation with lactic acid bacteria to best insure the desired results, especially when fodders are used which are not fit for a spontaneous lactic fermentation.

In conclusion it pleases me to be able to emphasize that the majority of my results have been verified in recent years by various authors, *i.e.*, Barthel, Boekhout, and De Vries, Burri, Esten, Evans, Hardings, Harrison, Hoffman, Löhnis, Orla Jensen, and others.

A METHOD FOR THE CULTIVATION OF ANAEROBES[1]

L. D. BUSHNELL

Kansas Agricultural Experiment Station

Received for publication September 11, 1921

In the course of a study of the bacteria causing spoilage in canned foods it became necessary to place large numbers of cultures under anaerobic conditions, and to devise anaerobic methods which would care for them with a minimum of expense of time and material.

A review of the literature showed that the most commonly recommended methods, which would be suitable for these purposes, were absorption of oxygen by alkaline pyrogallate and the replacement of air by an inert gas, usually hydrogen, or a combination of the two.

The chief objections to these methods are that they are difficult to apply, requiring complicated apparatus, are expensive and time consuming. We have encountered still another difficulty in that even the most carefully made containers are likely to leak air. In case of a leak, the pyrogallate solution is soon exhausted, or the hydrogen passes out very rapidly and is replaced by air. We have found that even the all-glass Novy jar would not maintain anaerobic conditions for twenty-four hours in many cases. These jars are also of rather limited capacity and very expensive. The number of methods suggested would lead one to doubt the complete suitableness of any one for all occasions, and experience with their use merely establishes the correctness of this impression.

Several years ago Dr. Wolbach suggested the method of Sellards which he had used successfully in his work on anaerobes.

[1] Contribution Number 36 from the Bacteriological Laboratories of the Kansas Agricultural Experiment Station.

This method makes use of metallic phosphorus for the removal of oxygen. Most authors mention this method of obtaining anaerobic conditions in their review of the literature, but so far as we have been able to find, none of them except Wolbach have advocated it. In the introduction to his article describing this method, Sellards (1904) makes the statement that up to that time no one had recommended the use of phosphorus for anaerobic work, although phosphorus is one of the most powerful absorbing agents of oxygen. Among the recommendations for its use, this author mentions that phosphorus is very convenient, that it requires no previous preparation, that it keeps well, and that its absorbing efficiency is very easily tested.

He mentions two possible difficulties; first, that the oxides of phosphorus formed during the absorption of oxygen might change the reaction of the medium employed; second, that the vapors of the elementary phosphorus might injure the nutrient media. He mentions not having had any difficulty from these sources.

The phosphorus method has been used in this laboratory for the past three years and we wish to recommend it for the cultivation of anaerobic bacteria. We have been able to culture all obligate anaerobes, which we have tried upon the surface of plates and slant agar. Obligate anaerobes also grow well in liquid media under anaerobic conditions produced by phosphorus. We have had no difficulty concerning the injury to the media. There is a slight rise in temperature within the jar as the phosphorus burns; this, however, exerts no detrimental effect upon the cultures. One great advantage of this method over others is that the power of phosphorus to absorb oxygen is so great that a rather small amount will absorb the oxygen within the jar, and enough will remain to absorb whatever may leak in during the incubation period. Sellards recommended the presence of an excess of alkali to absorb the phosphorus acid anhydrides. We have found that water serves the same purpose almost equally well. There is some theoretical objection to burning the phosphorus in the jar, in that elementary phosphorus is volatilized. We have had no difficulty from this source as far as the growth of bacteria is concerned, but some of the material is deposited

upon the surface of the plates and upon the cotton plugs, thus making the culture dishes unpleasant to handle. This has been overcome to some extent by covering the plates and cotton plugs with paper. Another source of trouble was encountered in labeling the tubes with a wax pencil. The phosphorus seemed to soften the wax of the ordinary red wax pencil, causing the labels to become blurred. Later a blue wax pencil was found which was not influenced in this way. We have also resorted to the use of paper labels held in place by rubber bands, since the excessive moisture in the jar will soon cause gummed labels to fall off.

Some difficulty was experienced in obtaining an anaerobic jar large enough to hold all the plates or cultures which we wished to run at one time, and at the same time, one which could be easily handled and remain air tight under a reduced pressure of three to four inches of mercury for several days. Quart "Lightning Seal" fruit jars have been used for test tube work to some extent. In this case, however, the sides of the jar and tubes must be protected from the burning phosphorus. This may be accomplished by strips of thin asbestos board.

As a large anaerobic jar, we have used an aluminum pressure food cooker (figs. 1 and 2), manufactured by the Pressure Cooker Company, of Denver, Colorado. The pressure gauge on the top is removed and this opening plugged. Just before using, the edge of the cover is heavily smeared with a rubber cement, similar to that used on stopcocks. This jar will retain a vacuum of 4 to 5 inches of mercury for as long as two weeks in the incubator at 37°C. (we have not tested it for longer periods.) Water is placed in the bottom of the jar, cultures are placed on the wire rack (fig. 2), an evaporating dish containing the phosphorus is placed in the jar and covered with a bit of wire gauze having a small opening in the center. This is to prevent the burning phosphorus from spattering. When all is ready, one of the stopcocks in the cover is opened and the cover is held so that it may be replaced quickly A hot needle is passed through the small hole in the wire gauze, and the phosphorus is ignited. The cover is quickly replaced and screwed down firmly. The pet cock is shut off after a few seconds, and a careful watch main-

tained for leaks. As the phosphorus burns there is considerable positive pressure due to the increase in temperature The pet cock should always be left open until this is nearly neutralized, otherwise leaks may develop. If there are leaks around the lid, they can be detected by the puffing out of phosphorus fumes. The burning usually ceases in a very few seconds, due to the exhaustion of oxygen; and the jar is filled with fumes. In glass jars these are seen to subside in about fifteen minutes if there is plenty of fresh water in the bottom. Anaerobic conditions are

FIG. 1. ANAEROBIC JAR WITH LID IN PLACE

FIG. 2. ANAEROBIC JAR WITH LID REMOVED

thus obtained about the cultures almost instantly. The rate at which oxygen dissolved in the medium, will diffuse out will depend upon the medium. Litmus milk and broth containing methylene blue are decolorized within a few hours. Deep agar tubes containing methylene blue are not decolorized to the bottom for about three days. Apparently oxygen diffuses out about as slowly as it diffuses into the media, since oxygen will diffuse into agar at the rate of about 1 cm. per diem. This is indicated by the fact that most vigorously growing anaerobes will grow to within one or 1.5 cm. of the surface of a deep agar tube.

The jar which we have described is of light weight, quickly sealed and very convenient. It may also be used with hydrogen or illuminating gas. If alkaline pyrogallate is used it must be

placed in a separate dish and not upon the bottom of the jar since these chemicals attack the metal. The capacity of the size which was used is about twenty-five plates or eighty test tubes of 15 mm. diameter. Everything considered, this is the most convenient and effective method of culturing anaerobes which we have yet encountered. One precaution which must be taken on all occasions, is not to allow bits of phosphorus to remain exposed to the air. These may not burn for several hours if moist, but as soon as they become dry they will burn and may set fire to the laboratory. The phosphorus in stock should be placed in firmly corked glass bottles and kept in the ice box. In this laboratory we have had some difficulty because the temperature became so high during the summer that the sticks melted together into a mass in the bottom of the container, thus making it difficult to remove. This trouble may be overcome by keeping the material in the ice box. Sellards recommends drawing out the phosphorus into small sticks. This is not at all necessary, and involves considerable trouble.

REFERENCE

SELLARDS, A. W. 1904 Some researches on anaerobic cultures with phosphorus. Cent. f. Bakt. Abt. I. **37**, 632-637.

INFLUENCE OF VACUUM UPON GROWTH OF SOME AEROBIC SPORE-BEARING BACTERIA[1]

L. D. BUSHNELL

Kansas Agricultural Experiment Station

Received for publication September 11, 1921

In the course of a study of the bacteria present in canned foods, we found that certain aerobic spore-bearing bacteria were present in jars which showed no evidence of spoilage, if they were properly sealed. These results were reported from this laboratory in 1918 (Bushnell 1918), but no attempt was made to determine the types present.

Vaillard, in 1900 and 1902 examined bacteriologically, many cans of meat, and found living organisms in seventy or eighty percent of them. He believed that the bacteria survived in a dormant state in the cans from five to seven years. Among the spore-bearing types he isolated *B. subtilis* and *B. mesentericus*, (three varieties, *vulgatus*, *ruber*, and *fuscus*).

Deichsetter, in 1901, reported on the examination of preserved food provided for the Bavarian Army during a period of five years and failed to find microörganisms in canned foods, save in cans in which the food was sent in under suspicion. He considered that Vaillard's findings were probably due to faulty technic.

Pfuhl, in 1904, examined canned meats from five firms and found bacteria in 29 out of 106 cans. He considered that the findings of both Vaillard and Deichsetter were correct and that the difference in results was due to a difference in the care with which the foods were sterilized.

Very little work had been reported upon this point until 1919 when Weinzirl published the results of his findings on commercial

[1] Contribution Number 37 from the Bacteriological Laboratory of the Kansas Agricultural Experiment Station.

canned foods. He states that in commercial canned foods giving no evidence of spoilage, microörganisms were found in 179 out of a total of 782 cans, or in 23 per cent of the cans. The spore-bearers were practically the only organisms present, due to their superior resistance to the sterilizing process. Viable spores were found in 19.2 per cent of the non-leaking cans. Of the types of bacteria isolated, *B. mesentericus* predominated, with *B. subtilis* next. This author concludes that the living spores in commercial canned foods are unable to grow, due to the absence of oxygen, and that the vacuum is essential to the preservation of canned foods under the present method of processing.

Cheyney, in 1919, reported *B. mesentericus* in apparently perfect cans, which were given a standard processing.

Hunter and Thom, in 1919, made an examination of 530 cans of canned salmon and found 237 unsterilized. 234 of these cans contained the same organism of the *B. mesentericus* group, either in pure culture or in connection with other species. Only 13 showed active spoilage.

From the above it is evident that the aerobic spore-bearing types predominating in unspoiled canned foods belong to the *B. mesentericus* and *B. subtilis* groups of bacteria.

We may consider three reasons why these organisms predominate in foods under such conditions:

1. Spores of certain types predominate on the product as it goes into the container.

2. Spores of certain types are more resistant to heat than spores of other types.

3. The spores of certain types are not all destroyed during the processing period and those remaining are able to grow under conditions as they exist in the container.

From the results obtained in this laboratory, it is evident that *B. mesentericus* predominates, with *B. subtilis* second in number among the aerobic spore-bearing types. We have no idea of the number of each type upon the raw product as it went into the jars, so that it is not possible to consider this point except in so far as we may apply the work of Bruett (1919) upon the death of bacteria. She concluded that the death rate followed

the laws of monomolecular reactions. According to this law, if all spores were of equal resistance to heat, those present in the largest number would be the last to disappear. However, spores of different species of bacteria are not of equal resistance to heat, and while those of a particular species may follow this law, it cannot be applied in a comparison of the thermal death rate of different species.

We have found that the several cultures of *B. mesentericus* with which we have worked, are less resistant than the strains of *B. subtilis*. Regardless of this decreased resistance *B. mesentericus* predominated among the organisms isolated. Our cultures had grown for some time upon culture media and the resistance may have changed by this treatment. Weinzirl considers that *B. mesentericus* predominates in canned foods because of its superior heat-resisting qualities.

Lawrence and Ford (1916) state that the spores of *B. subtilis* survive steaming one and one-fourth hours in the Arnold sterilizer and autoclaving up to and including 19 pounds pressure but are usually destroyed at 20 pounds. The *B. mesentericus* spores survived one hour in the Arnold sterilizer and autoclaving at 19 pounds pressure, being killed by 20 pounds pressure. These statements would indicate that their cultures of *B. subtilis* were somewhat more resistant than those of *B. mesentericus*.

From some previous work upon these two types we had considered that *B. mesentericus* could grow in the absence of oxygen more readily than *B. subtilis*. Cheyney in 1919, also calls attention to this fact in his recent article on the bacteriology of canned foods.

We have occasionally isolated *B. mesentericus* from the deeper layers of agar in our search for anaerobes, but we have never isolated *B. subtilis* under such conditions, although it has been found on the surface several times. It must be admitted that we did not make quantitative determinations of the types present in each jar. The predominance merely means that in the routine isolation of colonies more of the *B. mesentericus* type were isolated, although we are convinced that this organism did predominate in the jars.

In an attempt to determine why *B. mesentericus* predominated, we undertook some experiments, using these two types. The types used were isolated from jars of asparagus and were rapid spore formers, although they had been grown in the laboratory for more than a year.

The thermal death point of the spores of the *B. subtilis* culture used in these experiments was from ninety to one hundred and twenty minutes in steam at 98°C.; for *B. mesentericus* eighty to one hundred minutes. The time at which all are killed depends to some extent upon the numbers present.

For the experimental work, the organisms were grown upon plain extract agar from four to six days. The growth was scraped from the medium and suspended in a small amount of sterile normal saline. This was shaken in a heavy walled bottle with glass beads and filtered through sterile cotton to remove clumps. The suspension was heated at 80°C. for twenty minutes to kill the vegetative cells.

Experiment 1. In this experiment we wished to determine the influence of different amounts of air upon the growth of the organisms. The number of spores indicated in the table were added to tubes of extract broth, 0.50 per cent N/1 acid to phenolphthalein pH 5.9. In this case the column of air above the medium was about 5 cc. The tubes were exhausted to various points and sealed. The results are shown in table 1.

From this table we may conclude that there was slight growth of both organisms during fifty-one days incubation at room temperature in the less exhausted tubes.

Experiment II was conducted in order to determine the influence of varying amounts of salt and air upon the growth of *B. subtilis* and *B. mesentericus*. In this case, known amounts of air were left above the liquid.

An attempt was made to remove all possible traces of air from the medium. To do this, the tubes were partially filled with a known amount of broth to which varying amounts of salt had been added. The tubes were then drawn out to a slender neck, as close to the liquid as possible, and heated in a seamer for fifteen minutes. The tubes were next cooled in cold water, and

1 cc. of a suspension of spores added. The spores were suspended in salt solutions to correspond to that in the tube, so that the salt concentration was not changed. All the tubes were filled to a mark on the constriction, with broth containing corresponding amounts of salt. A certain per cent of this was then removed and the tubes exhausted and sealed at the mark. The tubes were then incubated at 37°C. for twenty-seven days and plates made.

TABLE 1

DAYS INCUBATION	B. SUBTILIS					B. MESENTERICUS				
	Number spores added	Tubes exhausted to following mm. Hg. on manometer				Number spores added	Tubes exhausted to following mm. Hg. on manometer			
		175	350	525	685		175	350	525	685
5	3110	2740	2220	3000	2280	1300	1830	1730	1370	1440
5	31	25	18	43	58	13	32	31	36	30
12	3110	1170	7000	1900	1000	1300	2100	2000	1090	640
12	31	69	60	16	71	13	31	18	19	36
19	3110	2300	3500	2500	8000	1300	3200	1830	1020	120
19	31	50	41	50	30	13	60	38	13	20
41	3110	3200	4000	5000	6000	1300	4000	1990	1260	260
41	31	49	29	11	12	13	90	72	27	8
51	3110	9000	7000	3200	5000	1300	4000	2460	1120	370
51	31	90	120	12	11	13	150	71	18	11

Since each tube had a slightly different amount of broth added, it was necessary to calculate how many organisms were present in each centimeter of liquid in the beginning. These numbers are in one column and the number at the end of the incubation period in a parallel column. The volume of air varied from 0.3 cc., in the tubes containing 1 per cent air to 1.5 cc. in the tubes containing 25 per cent air, each tube being of somewhat different volume from the others. The results are shown in tables 2 and 3.

These tables show the same as table 1, that there is some growth in these tubes. There is an interesting point relative to the action of increased amounts of salt. In every case, when the average is taken for all tubes in the same concentration of salt, there is an increase over that of a lower concentration. This point is somewhat more in evidence in connection with

TABLE 2

Influence of varying amounts of salt and air upon the growth of B. subtilis in broth

EXHAUSTED TO MM. Hg.	AIR	1 PER CENT NaCl		2 PER CENT NaCl		4 PER CENT NaCl	
		Bacteria added per cubic centimeter	Bacteria determined per cubic centimeter	Bacteria added per cubic centimeter	Bacteria determined per cubic centimeter	Bacteria added per cubic centimeter	Bacteria determined per cubic centimeter
	per cent						
0	5	167,000	100,000	149,000	190,000	175,000	320,000
	10	146,000	150,000	157,000	200,000	165,000	250,000
	25	152,000	117,000	151,000	240,000	151,000	210,000
50	5	146,000	210,000	151,000	102,000	157,000	320,000
	10	152,000	160,000	157,000	110,000	165,000	240,000
	25	141,000	180,000	149,000	110,000	162,000	270,000
100	5	149,000	111,000	151,000	160,000	162,000	180,000
	10	162,000	220,000	143,000	240,000	143,000	270,000
	25	162,000	220,000	146,000	260,000	149,000	
Average		153,000	163,000	150,000	179,000	158,000	258,000
175	1	113,000	129,000	98,000	28,000	104,000	164,000
	5	135,000	76,000	119,000	27,000	124,000	384,000
	10	135,000	112,000			117,000	104,000
	25	113,000	102,000	129,000	158,000	113,000	424,000
350	1	113,000	89,000	129,000	200,000	124,000	448,000
	5	123,000	78,000			117,000	342,000
	10	132,000	98,000	124,000	200,000	118,000	476,000
	25	123,000	55,000	104,000	348,000	107,000	688,000
525	1	113,000	37,000	114,000	152,000	108,000	444,000
	5	113,000	84,000	114,000	256,000	113,000	436,000
	10	118,000	126,000	129,000	240,000	104,000	428,000
	25	123,000	76,000	114,000	220,000	118,000	416,000
685	1	123,000	97,000	124,000	26,000	119,000	516,000
	5			119,000	44,000	113,000	458,000
	10	141,000	48,000	119,000	100,000	113,000	454,000
	25	135,000	145,000	132,000	160,000	114,000	472,000
Average		123,000	90,100	118,000	153,000	114,000	416,000

TABLE 3

Influence of varying amounts of salt and air upon growth of B. mesentericus in broth

EXHAUSTED TO MM. Hg.	AIR	1 PER CENT NaCl		2 PER CENT NaCl		4 PER CENT NaCl	
		Bacteria added per cubic centimeter	Bacteria determined per cubic centimeter	Bacteria added per cubic centimeter	Bacteria determined per cubic centimeter	Bacteria added per cubic centimeter	Bacteria determined per cubic centimeter
	per cent						
0	5	340,000	320,000	328,000	280,000	323,000	720,000
	10	307,000	220,000	314,000	310,000	323,000	750,000
	25	345,000	450,000	328,000	260,000	340,000	860,000
50	5	328,000	340,000	328,000	450,000	341,000	940,000
	10	340,000	260,000	328,000	440,000	341,000	870,000
	25	323,000	290,000	345,000	480,000	328,000	Broken
100	5	323,000	Broken	314,000	440,000	328,000	560,000
	10	328,000	210,000	292,000	290,000	314,000	630,000
	25	340,000	190,000	345,000	360,000	353,000	810,000
Average		331,000	285,000	324,000	367,700	332,300	767,500
175	1			15,300	35,000	17,400	23,000
	5	15,300	29,000	15,500	36,000	15,300	25,000
	10	14,900	24,000	17,700	36,000	15,300	45,000
	25	17,400	22,000	14,300	60,000	14,500	24,000
350	1	15,200	18,000			17,700	22,200
	5	16,000	11,000	15,900	26,000	17,400	24,000
	10	17,700	32,000	18,500	15,000	15,900	48,000
	25	18,100	31,000	15,700	34,000	17,700	29,000
525	1	16,600	26,000	15,300	27,000		
	5	17,600	26,000	14,900	32,000	17,400	24,000
	10	18,600	18,000	16,000	27,000	15,700	34,000
	25	16,000	27,000	16,200	22,000	16,600	29,000
685	1			17,400	29,000	16,000	25,000
	5	17,700	15,000	16,000	27,000	15,700	30,000
	10	16,000	13,000	18,600	30,000	17,400	24,000
	25	16,100	12,000	15,700	20,000	17,400	21,000
Average		16,600	21,500	16,900	30,400	16,700	31,300

B. mesentericus than with *B. subtilis*. The very small amount of air remaining in these tubes apparently has no influence upon the amount of growth. There were relatively no more organisms present in tubes merely sealed and with 25 per cent volume of air, that there were in those tubes which were exhausted and with but 1 per cent of the volume of air. We believe that the organisms in the tube exhausted to 685 mm. were under as completely anaerobic conditions as it is possible to obtain.

According to Bitting and Bitting (1916) an ordinary tin can shows a vacuum of about four inches of mercury when exhausted at a temperature of 130°F. and tested at 85°F.

We may conclude from the results obtained, that the degree of vacuum plays no part in the destruction of the spores of these two organisms. When one per cent of salt is present, there may be a slight decrease in the twenty-seven days of incubation. This is somewhat more marked in higher vacuum than in the lower, but the differences are not marked. In the presence of larger amounts of salt, there appears to be an actual increase in the number of viable bacteria. This is apparently not due to accidental conditions, since we have made numerous parallel determinations and find that the averages of those determined at the end of the incubation period are two or three times as high as the number added. Why there should be a decrease in the presence of 1 per cent, and an increase in the presence of 4 per cent salt we are unable to say. Of course there is considerable variation in the determinations, but the averages indicate a real increase.

Experiment III. This experiment was set up parallel with that of experiment II, except that varying amounts of acid were added to the broth. The organisms were treated in the same way as those in the last experiment, except that they were suspended in acid broth after heating to kill the vegetative forms. The tubes were incubated at 37°C. for twenty-three days for *B. subtilis* and twenty-two days for *B. mesentericus*. The results are shown in tables 4 and 5.

Apparently *B. subtilis* spores are more sensitive to acid than those of *B. mesentericus*. The degrees of vacuum had no influence

TABLE 4

Influence of varying amounts of acetic acid and air upon the growth of B. subtilis in broth

EXHAUSTED TO MM. Hg.	AIR	PER CENTS OF N/1 ACID AND pH							
		0.5 per cent pH 5.6		1 per cent pH 5.60		2 per cent pH 4.30		4 per cent pH 4.00	
		Added	Determined	Added	Determined	Added	Determined	Added	Determined
	per cent								
0	5	715,000	429,000	800,000	173,000	890,000	241,000	800,000	184,000
	10	783,000	450,000	858,000	219,000	872,000	205,000	890,000	170,000
	25	716,000	457,000	737,000	265,000	715,000	256,000	828,000	145,000
50	5	813,000	392,000	907,000	223,000	800,000	234,000	858,000	176,000
	10	872,000	465,000	737,000	232,000	828,000	247,000	858,000	166,000
	25	783,000	472,000	813,000	175,000	761,000		800,000	186,000
100	5	858,000	408,000	872,000	209,000	858,000	198,000	813,000	52,000
	10	813,000	430,000	858,000	250,000	847,000	236,000	761,000	100,000
	25	783,000	472,000	813,000	273,000	828,000	234,000	800,000	130,000
Average		802,000	443,000	836,000	224,000	822,000	231,000	823,000	146,000
175	5	409,000	256,000	410,000	214,000	410,000	157,000	391,000	
	10	383,000	250,000	451,000	270,000	409,000	171,000	391,000	70,000
	25	409,000	303,000	383,000	190,000	451,000	192,000	400,000	170,000
350	5	400,000	212,000	409,000	250,000	409,000		409,000	140,000
	10	374,000	270,000	429,000	230,000	440,000	148,800	409,000	103,000
	25	451,000	303,000	440,000	137,000	409,000	141,600	400,000	210,000
525	5	440,000	266,400	366,000	128,000	409,000	162,400	451,000	100,000
	10	383,000	259,400	429,000	178,000	463,000	146,400	391,000	180,000
	25	410,000	273,600	409,000	210,000	366,000	165,600	409,000	110,000
685	5	463,000	276,000	440,000	230,000	410,000	176,800	400,000	140,000
	10	400,000	258,000	391,000	220,000	488,000	162,400	429,000	190,000
	25	391,000	216,000	463,000	225,000	429,000	158,400	400,000	131,000
Average		409,400	262,300	418,000	206,900	424,400	162,100	407,700	140,000

upon the decrease in numbers, but the higher amounts of acid were more active than the smaller amounts. As with *B. subtilis* in the presence of salt, there is a marked decrease even in the tubes with smaller amounts, but unlike the higher amounts of

TABLE 5

Influence of varying amounts of acid and air upon the growth of B. mesentericus in broth

EXHAUSTED MM. Hg.	AIR	PER CENTS OF N/1 ACID AND pH							
		0.5 per cent pH 5.60		1 per cent pH 5.00		2 per cent pH 4.30		4 per cent pH 4.00	
		Added	Determined	Added	Determined.	Added	Determined	Added	Determined
	per cent								
0	5	116,000	90,000	114,000	120,000	114,000	240,000	109,000	120,000
	10	104,000	80,000	114,000	150,000	102,000	220,000	102,000	130,000
	25	102,000	89,000	102,000	140,000	118,000	280,000	101,000	230,000
50	5	108,000	54,000	108,000	140,000	102,000	240,000	106,000	140,000
	10	111,000	56,000	96,000	68,000	118,000		91,000	150,000
	25	104,000	75,000	104,000	160,000	108,000		111,000	150,000
100	5	111,000	45,000	114,000	110,000	91,000	120,000	96,000	113,000
	10	101,000	52,000	109,000	110,000	109,000	190,000	102,000	113,000
	25	109,000	48,000	104,000	200,000	102,000		108,000	140,000
Average		107,000	66,000	107,000	199,000	107,000	215,000	103,000	151,000
175	5	40,000	19,200	42,900	16,800	47,600	18,000	38,300	49,600
	10	41,000	16,000	33,200	22,800	45,100	20,000	37,400	33,600
	25	47,600	19,200	36,000	23,200	39,000	36,000	42,900	47,200
350	5	42,900	14,800	36,600	25,200	42,900	49,000	46,300	36,800
	10	44,000	15,200	45,100	26,800	40,900	35,000	46,300	48,000
	25	40,000	12,000	41,000	19,200	46,300	60,000	45,100	65,600
525	5	40,000	14,400	40,900	14,000	36,100	28,000	41,000	43,300
	10	41,000	16,500	44,000	16,800	48,800	30,000	38,300	59,200
	25	39,000	16,000	39,100	14,000	47,600	27,000	37,400	40,000
685	5	44,000	16,000	41,000	18,400	45,100	17,000	39,100	48,000
	10	48,800	18,800	40,000	21,600	45,100		46,300	43,000
	25	45,000	12,800	40,000	13,600	45,100	34,000	44,000	51,200
Average		42,800	15,980	40,300	19,360	44,100	31,700	41,800	47,140

salt there is a marked decrease in the higher concentrations of acid. Even sealing seems to cause a marked decrease in the number of viable spores of both types. Perhaps if the incubation period had been lengthened there would have been a still greater

decrease in case of *B. subtilis*. Parallel tubes which were not sealed showed heavy growth of these organisms in all concentrations of salt, but only a trace of growth in the higher amounts of acid. In the sealed tubes there was a very faint visible trace of surface growth in the tubes containing larger amounts of air. This was easily broken up and did not re-form on standing. The plates made from these tubes checked much better than would generally be expected, when it is considered that organisms of this type produce such heavy surface growths in open tubes. However, these organisms do not form such adherent growths in sealed tubes, and by vigorous shaking a fairly uniform suspension may be obtained.

In the case of *B. mesentericus* there was much less marked action of acid. Either there is not so much decrease in the higher amounts of acid or there is a slight increase of this organism after the initial decrease. At the end of the incubation period there were about the same numbers as at the beginning of the experiment.

In *Experiment IV* an attempt was made to determine the influence of the amount of air and salt upon the thermal death point of *B. subtilis* and *B. mesentericus*. The results are shown in tables 6 and 7. The tubes and spore suspensions were prepared as above.

It is evident from these tables that *B. mesentericus* spores are somewhat more easly killed by heat than those of *B. subtilis*. We have found this to be true in numerous other tests upon the thermal death point of these organisms.

The amount of salt or the amount of air has practically no influence upon the thermal death point, particularly in the case of *B. subtilis*, the larger amounts of salt seeming, however, to protect the organisms to some extent.

Experiment V shows the influence of acetic acid and varying amounts of air upon the thermal death point of *B. subtilis* and *B. mesentericus*.

In this experiment the spores were prepared as above described. The liquid in which the spores were suspended during the heating was extract broth plus 0.5 percent; 1 per cent; 2 per cent; 4 per

cent of N/1 glacial acetic acid, giving the pH as shown in the tables.

Two centimeters of this acid broth were added to the tubes which were then heated in the steamer for fifteen minutes and cooled as rapidly as possible to remove air from the liquid. To

TABLE 6

Influence of varying amounts of salt and air upon thermal death point of B. subtilis spores. Original numbers of spores added 32,000,000 per cubic centimeter

SALT	MM. Hg.	TIME OF HEATING AT 98°C.			
		15 minutes	30 minutes	60 minutes	120 minutes
per cent					
1	Open	460,000	140,000	6,100	15
	175	110,400	40,000	7,600	31
	350	520,000	170,000	6,500	49
	525	640,000	200,000	8,300	15
	685	400,000	121,000	9,300	32
Average		426,000	134,000	7,560	28
2	Open	670,000	180,000	8,700	38
	175	500,000	250,000	9,800	21
	350	400,000	120,000	6,400	42
	525	580,000	210,000	14,600	71
	685	700,000	160,000	10,400	24
Average		570,000	184,000	9,980	39
4	Open	860,000	480,000	18,000	36
	175	840,000	200,000	14,500	56
	350	840,000	190,000	33,600	66
	525	473,000	200,000	26,000	82
	685	860,000		44,000	72
Average		774,600	302,500	27,200	62
General average		590,200	206,800	14,910	44

these tubes was added 1 cc. of a heavy suspension of spores suspended in acid broth, similar to that in the tubes. The tubes were then filled to the mark with similar broth and the volume noted. Amounts equal to 5 per cent, 10 per cent, 25 per cent of the total volume were removed from the tubes. They were

then exhausted to the points desired and sealed at the mark. The tubes were all placed in a steam sterilizer and heated for one hour. Tables 8 and 9 show the results obtained. Since each tube was of slightly different volume the liquid remaining after removing the above amounts would contain slightly different

TABLE 7

Influence of varying amounts of salt and air upon the thermal death point of B. mesentericus spores. Original number of spores added 3,780,000

SALT	MM. Hg.	TIME OF HEATING AT 98°C.			
		15 minutes	30 minutes	60 minutes	120 minutes
per cent					
1	Open	8,000	700	17	2
	175	2,400	200	8	0
	350	1,000	130	34	0
	525	3,600	500	17	0
	685	1,600	200	8	0
Average		3,320	346	17	1
2	Open	2,300	1,100	98	0
	175	1,100	800	30	0
	350	1,400	400	27	0
	525	1,000	520	39	0
	685	1,000	700	74	0
Average		1,360	704	53	0
4	Open	1,700	390	69	0
	175	1,000	640	56	0
	350	2,300	700	88	3
	525	1,400	320	20	0
	685	1,500	200	70	0
Average		1,580	450	61	1
General average		2,087	500	44	1

numbers per cc. The reduction in numbers in this experiment is so striking that the numbers are not included in the tables in each case. The average number for each cc. of the liquid remaining in the tubes was about 37,400,000 per centimeter for *B. subtilis* and 1,760,000 for *B. mesentericus*.

The above tables show a very marked influence of acid upon the thermal death point of both organisms. The fact that canned fruits and vegetables containing, or treated with acid, kept so much better than fruits and vegetables containing no acid, was

TABLE 8

Influence of varying amounts of acetic acid and air upon the thermal death point of of B. subtilis spores

MM. Hg.	AIR	PER CENTS OF N/1 ACETIC ACID			
		0.5 per cent pH 5.5	1 per cent pH 5.10	2 per cent pH 4.5	4 per cent pH 4.10
	per cent				
175	5	590	9	3	0
	10	490	0	0	0
	25	700	0	2	0
Average		593	3	1	0
350	5	482	0	0	0
	10	142	0	2	0
	25	336	0	6	0
Average		320	0	2	0
525	5	43	0	0	0
	10	113	0	0	0
	25	46	0	0	0
Average		67	0	0	0
685	5	44	0	2	1
	10	600	3	4	2
	25	702	1	0	0
Average		448	1	2	1
Open		372	0	3	1
General average		360	1	2	1

formerly thought to be due to the fact that the acid inhibited growth. From the results which we have obtained, we are inclined to believe that the keeping is due, not so much to the influence of the acid in inhibiting growth as to the fact that most or all of the organisms present are killed by the heating process.

The few remaining are probably unable to grow to any extent in the highly acid medium.

Here, also, the amount of air present in the containers has no influence whatever upon the thermal death point of the bacteria present.

TABLE 9

Influence of varying amounts of acetic acid and air upon the thermal death point of B. mesentericus spores

MM. Hg.	AIR	PER CENTS OF N/1 ACETIC ACID			
		0.5 per cent pH 5.0	1 per cent pH 5.10	2 per cent pH 5.50	4 per cent pH 4.10
	per cent				
175	5	4	1	0	0
	10	10	1	0	0
	25	8	0	0	0
Average		7	1	0	0
350	5	11	0	0	0
	10	2	1	0	0
	25	16	1	0	0
Average		9	1	0	0
525	5	13	2	0	0
	10	10	1	0	0
	25	10	3	0	0
Average		11	2	0	0
685	5	2	2	0	0
	10	5	0	0	0
	25	16	2	0	0
Average		7	1	0	0
Open		12	2	0	0
General average		9	1	0	0

Experiment VI shows the influence upon the thermal death point of *B. subtilis* and *B. mesentericus* of several of the more common organic acids found in fruits. The spores were prepared as described in experiment V and placed in the acid solutions

after heating at 80°C. for twenty minutes to kill the vegetative forms. The results are shown in table 10.

TABLE 10

Influence of organic acids upon the thermal death point of B. subtilis and B. mesentericus spores

	TIME OF HEATING	PER CENT OF N/1 ACID ADDED TO BROTH			
		0.5 per cent	1 per cent	2 per cent	4 per cent
B. subtilis added 7,305,000 per cubic centimeter					
	minutes				
Lactic	15	74,400	17,300	3,200	273
	30	3,400	2,000	50	0
	60	80	10	0	0
B. mesentericus added 2,500,000 per cubic centimeter					
Lactic	15	18,000	100	90	10
	30	8,200	0	0	0
	60	13	0	0	0
B. subtilis added 17,500,000 per cubic centimeter					
Tartaric	15	520,000	410,000	52,000	2,000
	30	73,000	54,000	3	3
	60	600	576	2	0
B. mesentericus added 2,145,000 per cubic centimeter					
Tartaric	15	14,000	1,630	4	10
	30	1,200	220	0	0
	60	39	3	0	0
B. subtilis added 27,000,000 per cubic centimeter					
Citric	15	2,000,000	110,000	200	500
	30	240,000	600		200
	60	1,200	4	4	0
B. mesentericus added 17,000,000 per cubic centimeter					
Citric	15	24,000	60	50	12
	30	2,150	48	0	0
	60	0	0	0	0

The results show that there is very little difference in the action of these organic acids. The acetic is perhaps a little more effec-

tive, but not enough to be of practical importance. This table also shows the influence of the amount of acid, and also the fact that *B. mesentericus* is somewhat more easily destroyed by heat than *B. subtilis*.

SUMMARY

From the results obtained, we are inclined to believe that *B. mesentericus* predominates in canned foods because it is capable of growing to some extent in absence of air, rather than because its spores are more heat resistant than some other types of aerobic bacteria.

The amount of vacuum under which spores of these organisms are placed during the heating does not influence the thermal death point.

The small amount of acid present had but slight retarding influence upon the growth of these organisms in air, but did have a marked influence upon the thermal death point. It may be that the beneficial influence of acid upon the keeping of canned foods is due more to the lowering of the thermal death point than to the inhibition of growth of the organisms.

The amount of air remaining above the liquid has little influence upon the growth of these bacteria, since sealing the tubes prevents all but minimum growth. This inhibiting influence is more marked in case of *B. subtilis* than in case of *B. mesentericus*.

REFERENCES

BITTING, A. W., AND BITTING, K. G. 1916 Canning and how to use canned foods. Bull. 14, National Canners Assoc., Washington, D. C., 191 pp.

BRUETT, E. M. 1919 Utility of blanching in food canning. Effect of cold shock upon the bacterial death rates. Journ. of Ind. & Eng. Chem. **11**, 37–39.

BUSHNELL, L. D. 1918 The influence of cold shock in the sterilization of canned foods. Jour. of Ind. & Eng. Chem. **6**, 431–436.

CHEYNEY, E. W. 1919 A study of the microörganisms found in merchantable canned foods. Jour. of Med. Res., **40**, 177–197.

DEICHSETTER, J. 1901 Ueber den Keimgehalt den Fleischkonserven. Zeitschrift für Untersuch, d. Nahr und Genussmit., **4**, 1115.

HUNTER, A. C., AND THOM, C. H. 1919 An aerobic spore-forming bacillus in canned salmon. Jour. Ind. & Eng. Chem., **11**, 655–657.

LAWRENCE, J. S., AND FORD, W. W. 1916 Studies on aerobic spore-bearing non-pathogenic bacteria. Jour. Bact. **21**, 273–320.

PFUHL, E. 1904 Beiträge zur Bakteriologischen Untersuchungen den Fleischkonserven, Zeitschrift für Hygiene. **48**, 121–134.

VAILLARD, L. 1900 Les Conserves, Rept. to 10th Congress of Hygiene and Dem., Paris.

VAILLARD, L. 1902 Les conserves de la viande: Les accidents qu'elles provoquent leurs causes; les moyens de les prevenir. Rev. d'Hygiene, **24**, T. 17–35 and 109–121.

WENZIRL, J. 1919 The bacteriology of canned foods. Jour. of Med. Res. **39**, (New Series 34) 349–413.

SUBSTITUTION OF BROM-THYMOL-BLUE FOR LITMUS IN ROUTINE LABORATORY WORK

H. R. BAKER

Contribution No. 44 from the Department of Bacteriology, Kansas State Agricultural College

Received for publication December 10, 1921

One of the common methods for the qualitative determination of acid or alkali production by bacteria is to inoculate nutrient extract broth containing various carbohydrates with litmus as an indicator.

Litmus possesses the disadvantage of being reduced by many organisms to a colorless compound, thus rendering it useless as an indicator. Clark and Lubs (1917) mention that the sulphonphthalin indicators are much more resistant to bacterial action than indicators like methyl red or litmus. They suggest, because of certain preliminary tests, that indicators like brom-thymol-blue and brom-cresol-purple might be used to advantage in replacing other indicators which are now used in making indicator media.

With this suggestion in mind, an experiment was undertaken to find a method of preparing media to determine qualitatively acid or alkali production by bacteria, which would be easy to prepare, and more sensitive than litmus; and one in which the reaction could be quickly determined at any time during the incubation period.

In this experiment, sugar free broth was used, which was prepared as follows:

One pound of ground lean meat was digested for two hours with 1 liter of distilled water. After cooking, the broth was filtered through absorbent cotton into a flask and sterilized in the autoclave at 18 pounds pressure for twenty minutes. When cold the broth was inoculated with a culture of *Bact. saccharolyte* (Rivas)

and incubated at 37°C. for forty-eight hours to render the medium sugar-free. Then the medium was sterilized in the Arnold for twenty minutes; 10 grams of peptone and 5 grams of sodium chloride were added; the reaction was adjusted to pH 7.0 with brom-thymol-blue; the medium was again steamed for twenty minutes, the reaction readjusted, and the medium filtered.

To determine the amount of brom-thymol-blue which would inhibit acid production by microorganisms, fifty cubic centimeters of sugar free broth were placed in each of fifteen flasks.

TABLE 1

The influence of brom-thymol-blue upon acid production by Bact. coli-communis. Using 1 per cent glucose broth, initial pH 7.0, increasing amounts of a 0.2 per cent alcoholic solution of brom-thymol-blue inoculated with 0.1 cc. of an eighteen hour broth culture

LENGTH OF INCUBATION	DILUTIONS OF BROM-THYMOL-BLUE														
	0	1:125,000	1:62,500	1:41,666	1:31,250	1:25,000	1:20,750	1:17,810	1:15,610	1:13,900	1:12,500	1:11,360	1:10,420	1:9,650	1:8,875
hours															
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2½	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
3	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+
4	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++

− = No acid production; + = moderate acid production; ++ = strong acid production.

To each flask was added sufficient 0.2 per cent alcoholic solution of brom-thymol-blue to give the concentrations as shown in the tables. Equal amounts of the media were placed in five test tubes of similar size, and the tubed media were sterilized in the autoclave at 18 pounds pressure for twenty minutes.

When the tubes were cold, one cubic centimeter of a sterile 20 per cent glucose solution was added, under aseptic conditions, to each tube. The tubes were incubated at 37°C. for forty-eight hours, in order to make certain that none of them became contaminated during the process of adding the glucose solution.

TABLE 2

The influence of brom-thymol-blue upon acid production by Bact. typhosum, using media and dilutions as in table 1

LENGTH OF INCUBATION	DILUTIONS OF BROM-THYMOL-BLUE														
	0	1:125,000	1:62,500	1:41,666	1:31,250	1:25,000	1:20,750	1:17,810	1:15,610	1:13,900	1:12,500	1:11,360	1:10,420	1:9,650	1:8,875
hours															
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−
5	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
6	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+

One series of the tubes was inoculated with 0.1 cubic centimeter of an eighteen-hour culture of *Bact. coli-communis;* the second set was inoculated with the same amount of *Bact. typhosum;* the third with *Staphylococcus albus;* the fourth with *Bacillus subtilis;* and the fifth series was left as a check. All five sets of tubes were incubated at 37°C.

The data in the tables show that brom-thymol-blue did not have a marked effect of inhibition on the acid production by the Gram negative *Bact. coli-communis* or *Bact. typhosum* and inhibited the two Gram positive organisms only in concentrations

TABLE 3

The influence of brom-thymol-blue upon acid production by Staphylococcus albus, using media and dilutions as in table 1

LENGTH OF INCUBATION	DILUTIONS OF BROM-THYMOL-BLUE														
	0	1:125,000	1:62,500	1:41,666	1:31,250	1:25,000	1:20,750	1:17,810	1:15,610	1:13,900	1:12,500	1:11,360	1:10,420	1:9,650	1:8,875
hours															
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−
6	++	++	+	+	+	+	+	+	+	+	+	+	+	−	−
7	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+

which were much higher than need to be used in actual acid determinations.

Bact. paratyphosum A, *Bact. dysenteriae*, *Bact. sanguinarium*, *Bact. pullorum*, and *Bact. enteritidis* were also tested out in the same dilutions shown in the tables. Results were obtained closely approximating those for *Bact. typhosum*.

It has been found by experience that a 1:41,666 solution of brom-thymol-blue gives the most desirable concentration for colorimetric comparison. The data in the tables show that this concentration can be used in the media without inhibiting the

TABLE 4

The influence of brom-thymol-blue upon acid production by Bacillus subtilis, using media and dilutions as in table 1

LENGTH OF INCUBATION	DILUTIONS OF BROM-THYMOL-BLUE													
	0	1:125,000	1:62,500	1:41,666	1:31,250	1:25,000	1:20,750	1:17,810	1:15,610	1:13,900	1:12,500	1:11,360	1:10,420	1:9,650	1:8,875
hours															
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
4	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
5	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−
6	++	++	++	+	+	+	+	+	+	+	+	+	+	−	−
7	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+

production of acid. This dilution is easily made by adding 12 cc. of a 0.2 per cent alcoholic solution of the indicator to every liter of sugar free broth before it is put into the fermentation tubes.

Media prepared in this way are now used in this laboratory by the students, and have been found successful for all their qualitative acid production tests.

It has also been used by a member of the department in determining acid production by a certain bacterium, upon about twenty carbohydrates. In this particular instance, readings of the reaction of the cultures were made every day over a period of four weeks of incubation.

The advantages of this medium are:

1. Brom-thymol-blue includes the neutral point in its range of hydrogen-ion concentration, so that the medium can be adjusted to exact neutrality before being inoculated.

2. A medium containing sufficient brom-thymol-blue to act as an indicator will not inhibit acid production.

3. Brom-thymol-blue is not reduced by microbial action.

4. The reaction of a carbohydrate medium containing brom-thymol-blue can be recorded at any time during incubation.

5. Changes in color with slight changes in hydrogen-ion concentration are more marked with brom-thymol-blue than with litmus.

6. Brom-thymol-blue is easier to prepare than litmus.

7. Heat does not affect brom-thymol-blue during sterilization.

8. The reaction of carbohydrate broth containing brom-thymol-blue can be read by artificial light, but this is impossible with litmus.

THE USE OF DOMESTIC METHYLENE BLUE IN STAINING MILK BY THE BREED METHOD

W. A. WALL AND A. H. ROBERTSON

New York Agricultural Experiment Station, Geneva, New York

Received for publication December 15, 1921

Some difficulty has been experienced in staining preparations of milk by the Breed method. This is well summarized in the Standard Methods for the Bacteriological Examination of Milk,[1] as follows: "Some methylene blue now on the market in powder form is very unsatisfactory in that solutions will dissolve the milk films, or will wash them with an even blue color in which the bacteria fail to show distinctly. Old or unfiltered stains are to be avoided as they may contain troublesome precipitates."

During the past summer an effort was made at this laboratory to find means to correct these difficulties. To this end five samples of methylene blue, all reported to dissolve the milk films, were obtained from H. J. Conn, Chairman of the Committee on Technique of the Society of American Bacteriologists. All proved unsatisfactory when the stain was made up in a saturated aqueous solution using distilled water, and irregular results were obtained upon making up the stain with tap water. It was found that the addition of a small amount (approximately a quarter gram per liter) of $CaCO_3$ in the form of precipitated chalk caused one of the samples to stain without dissolving the films, and the difficulty with another sample was corrected after the $CaCO_3$ had stood in the solution for 48 hours. On account of the poor solubility of the $CaCO_3$, Na_2CO_3 was tried with uniformly good results. $NaHCO_3$ seems to work equally well. This indicates that the addition of small amounts of Na_2CO_3 to aqueous solutions of those methylene blues which are worthless because they

[1] Third edition, 1921, p. 15, Amer. Pub. Health Assoc., Boston.

dissolve the milk preparations, may render them satisfactory for use.

Methylene blue for bacterial stains comes on the market both as a zinc double salt and as a zinc-free hydrochloride. Methylene blue "for bacilli" is generally the zinc salt but may contain various quantities of the free hydrochloride. The pure hydrochloride is a dark greenish-blue crystal and is handled under the trade names of "Medicinal" or "U. S. P." A solution of the pure hydrochloride usually gives a dark blue stain to dried films of milk which becomes deeper the longer the preparation is left in the solution. The deep blue of the casein can be taken out by decolorizing with alcohol. The bacteria do not lose their stain as rapidly as the casein, thus making it possible to prepare a good preparation with this form of methylene blue. Methylene blue "for bacilli," which may be the pure zinc salt but may contain certain amounts of the free hydrochloride, may be any color from a greyish-red to a reddish-blue. An aqueous solution of the zinc salt will stain satisfactorily; but it is insoluble in alcohol and cannot be used in the Loeffler formula. Usually decolorization with alcohol is unnecessary with zinc salt solutions. An aqueous solution of the zinc salt which contains very little or none of the pure hydrochloride may possibly cause the washed out appearance mentioned in the Committee report.

Aqueous staining solutions frequently become grossly contaminated with bacteria and molds, so much so that organisms from this source may be found on the smears stained in these solutions. To avoid this difficulty Loeffler's alkaline methylene blue has been tried with uniformly good results. Later it was found that any solution of methylene blue prepared in 30 per cent alcohol was as satisfactory as the Loeffler's formula, and also prevented the growth of organisms in the staining solution. These results indicate that stains prepared in alcoholic solutions are to be preferred to the aqueous solutions recommended in the Standard Methods Report; but on account of the insolubility of the zinc salt in alcohol, either the medicinal methylene blue or some methylene blue "for bacilli" which is not largely made up of the zinc salt must be used.